Telithromycin; The First Ketolide, The Interactions and Alterations

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ABSTRACT

Respiratory tract infections are a serious cause of morbidity and mortality throughout the world. The most frequent causes of infection are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and atypical pathogens such as *Chlamydophila pneumoniae*. These infections are treated traditionally with antimicrobial agents, mainly the penicillins and macrolides. However, the incidence of macrolide resistance has increased markedly over the past 10 years, particularly in France, Belgium and Italy. Therefore, new compounds like telithromycin, an erythromycin derivative called a ketolide (K), have been developed to overcome this problem.

In this study, clinical isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were tested *in vitro* against a range of antimicrobial agents, including telithromycin, to investigate the efficacy of this new drug. Telithromycin showed excellent activity against *S. pneumoniae*, including macrolide resistant strains, but its activity was not as high against *M. catarrhalis* or *H. influenzae*. For *M. catarrhalis* the minimum inhibitory concentration (MIC) of telithromycin was similar to that of the macrolides. With regard to *H. influenzae* telithromycin had higher activity than erythromycin and clarithromycin.

The *ermB* and *mefA/E* genes mediate resistance to macrolides in *S. pneumoniae* in most cases. The *ermB* gene confers resistance to macrolide, lincosamide and streptogramin B (MLS_B) antimicrobial agents by methylation of part of their

ribosomal binding site. The *mefA/E* gene mediates efflux of 14- and 15-membered macrolides. Recent investigations have also implicated mutations in the 23S rRNA site of MLS_BK interactions with macrolide and, in certain cases, ketolide resistance. Alterations of two ribosomal proteins L4 and L22 have also been associated with increased MLS_BK MICs and resistance.

In this study *in vitro* mutants were generated from three *S. pneumoniae* strains, 02J1095 (*ermB* positive), 02J1175 (*mefA/E* positive) and NCTC 13593 (MLS_BK sensitive), on telithromycin. The MICs of the final generation mutants of each parent were increased in comparison to the parent but only the mutants from the macrolide resistant parents, 02J1095 and 02J1175, were telithromycin resistant. In order to ascertain the mechanism used by these telithromycin resistant mutants to achieve resistance, the *ermB* gene and upstream region, the *mefA/E* gene, the 23S rRNA genes encoding domains II and V and the L4 and L22 riboprotein genes were amplified by PCR and sequenced. No alterations were located in any of the genes of the 02J1175 mutants investigated. No changes were present in the *ermB* genes or upstream regions, the 23S rRNA genes or the L4 or L22 riboprotein genes of the 02J1095 mutants except J III 8. J III 8 had a telithromycin MIC of >32mg/L and was a second-generation mutant of 02J1095. In this strain two mutations were present. The first was a $_{94}K$ to Q_{94} amino acid mutation in the L22 riboprotein. The second was a 208 base pair deletion in the upstream region of the *ermB* gene containing the control peptide and one of two ribosome-binding sites. This region controls the expression of the *ermB* gene and hence methylase production. These mutations either alone or together were not present in any other mutant. They have not been described

previously in *S. pneumoniae*. These mutations, while novel, do not alone explain the development of telithromycin resistance in *S. pneumoniae*. They do however; give an insight into how telithromycin interacts with the ribosome and the potential mechanisms clinical isolates may develop when telithromycin is introduced into the community.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Fiona Walsh.

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ABBREVIATIONS

Amoxn	Amoxycillin
Amoxv	Amoxiclav
Azith	Azithromycin
bp	base pair
CCCP	Carbonyl cyanide *m*-chlorophenyl-hydrazone
Cipro	Ciprofloxacin
Clar	Clarithromycin
Clind	Clindamycin
CO_2	Carbon dioxide
Ery	Erythromycin
Farop	Faropenem
Gemi	Gemifloxacin
Levo	Levofloxacin
Linez	Linezolid
MIC	Minimum inhibitory concentration
Moxi	Moxifloxacin
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTI	Respiratory tract infection
SDW	Sterile distilled water
Telith	Telithromycin
v/v	Volume/volume

"As long as man has lived on earth, disease has plagued him. Sickness is associated with life, and man everywhere endeavours to deal with it as best he can"

(Rosen, 1965)

Chapter One

Introduction

1.1 Bacterial Respiratory Tract Infections

Community acquired respiratory tract infections (RTI) are among the most prevalent infectious diseases in the developed world and are associated with a considerable healthcare burden (Murray *et al.*, 1997). Respiratory tract infections can affect both the upper and lower respiratory tract. Of all RTIs, about one third are thought to involve the lower respiratory tract and these infections are a major cause of death globally, being responsible for 4.3 million premature deaths in 1990 worldwide (Murray *et al.*, 1994) and pneumonia is the sixth most common cause of death in the US (Bartlett *et al.*, 2000). Although upper respiratory tract infections such as sinusitis, tonsillopharyngitis and otitis media are not generally life-threatening, they can have serious effects if left untreated. Lower RTIs; such as community acquired pneumoniae (CAP) and acute bacterial exacerbations of chronic bronchitis (AECB) are associated with morbidity and mortality.

Up to 80% of community-acquired RTIs are caused by one of three bacterial pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (Felmingham, 2002). Other important RTI pathogens include *Streptococcus pyogenes*, *Staphylococcus aureus*, anaerobic species and the atypical

pathogens: *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila pneumoniae*. While these bacterial pathogens are implicated in RTIs, it is also important to remember the viral RTI pathogens such as influenza virus, respiratory syncytical virus, adenovirus and parainfluenza virus.

The most common cause of CAP is *S. pneumoniae* and this is the pathogen most frequently associated with poor outcome and mortality. *Streptococcus pneumoniae* is also the most frequently reported pathogen in acute otitis media and sinusitis and is reported in a significant proportion of AECB cases. *Haemophilus influenzae* is the primary bacterial pathogen of AECB causing 30% to 50% of bacterial exacerbations, and is also associated with a high proportion of CAP, acute sinusitis and acute otitis media (Felmingham, 2002). *Moraxella catarrhalis* is identified in a significant proportion of patients with acute otitis media, acute sinusitis and AECB, and is particularly prevalent in immunosuppressed and hospitalised patients.

Atypical and intracellular pathogens such as *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila pneumoniae* are increasingly being recognised as important aetiological agents of RTIs, particularly CAP. Unlike other atypical infections *C. pneumoniae* infection affects adults of all ages, usually as a co-pathogen, and has been associated with severe infection and occasionally death (Lim *et al.*, 2001).

1.1.1 *Haemophilus influenzae* and *Moraxella catarrhalis*

Haemophilus influenzae and *M. catarrhalis* are aerobic commensal bacteria of the throat and nasopharynx. *Haemophilus influenzae* is a gram-negative rod and *M. catarrhalis* gram-negative diplococci. They are both opportunistic pathogens of the lung and are implicated in diseases such as otitis media, sinusitis, pneumonia, meningitis and bronchitis. *Haemophilus influenzae* is the commonest pathogen of acute exacerbations of chronic bronchitis.

Until the late 1970s, isolates of both species were highly susceptible to ampicillin and amoxycillin (Livermore, 1995). Nowadays, the activity of aminopenicillins without a ß-lactamase inhibitor has essentially been lost against *M. catarrhalis* and *H. influenzae* due to the acquisition of ß -lactamases by both bacteria. The ß-lactamase activity also compromises the use of some cephalosporins classes. *Moraxella catarrhalis* has thus far not been found to have high levels of resistance to antibiotics such as macrolides, cephalosporins, tetracyclines, ß-lactamase inhibitor combinations, fluoroquinolones and co-trimoxazole. Occasionally *M. catarrhalis* strains resistant to these agents do appear, most frequently tetracycline resistant (Roberts *et al.*, 1990). In *H. influenzae* resistance to tetracycline, chloramphenicol, sulphonamides, trimethoprim and quinolones also occurs (Powell *et al.*, 1991). The numbers of *H. influenzae* strains resistant to ampicillin by mutations in the penicillin binding proteins, rather than producing ß-lactamases, are increasing (Ubukata *et al.*, 2001).

1.1.2 *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*)

Chlamydophila pneumoniae is an obligate intracellular pathogen, which has been associated with CAP, bronchitis, pharyngitis, sinusitis, myocarditis, endocarditis and atherosclerotic cardiovascular disease (Grayston *et al.*, 1990). It was first isolated in 1965, but was specifically described as *C. pneumoniae* in 1989 by Grayston *et al.* Tetracycline or erythromycin are currently used to treat infections.

Resistance to antimicrobial agents has not been described either in clinical strains of *C. pneumoniae* or laboratory generated mutants to date. This is due to their unique biphasic life cycle and difficulties that it poses to culture the organism *in vitro*. The current diagnostic methods of PCR or ligase chain reaction also do not permit susceptibility testing. Even though resistance has not been reported in *C. pneumoniae* there is good reason to suspect that resistance does occur. Hammerschlag *et al.*, (1992) described 5 patients with culture-positive *C. pneumoniae* infections who had multiple positive cultures over several months despite appropriate antibiotic therapy.

1.1.3 *Streptococcus pneumoniae*

1.1.3.1 Historical Perspective

Sternberg in the USA and Pasteur in France first isolated *Streptococcus pneumoniae* simultaneously in 1880. In the 1880s the pneumococci was also shown to be capable of causing meningitis, endocarditis, arthritis and otitis media and experimental pneumococcal endocarditis in rabbits (Reviewed by Austrian, 1999). Following the isolation and identification of *S. pneumoniae* a number of important scientific advances were made: The recognition by Klemperers of the protective value of antiserum against infection with the homologous organism and the observation of the lytic bile effect on pneumococci by Neufeld in 1900. Recognition of serologically different types of *S. pneumoniae* in 1910 by Neufeld and Haendel led to specific antisera and thus to the first effective treatment for pneumococcal pneumonia (Reviewed by Austrian, 1999). Thereafter followed the observations of Avery, Heidelberger and Goebel on the chemical structure of structural antigens and their role in bacterial virulence. Another accolade of *S. pneumoniae* history was the genetic transformation of pneumococci by DNA, initiated by Griffiths in 1928 (Griffiths, 1928), and elucidated by Avery, MacLeod and McCarty in 1944 (Avery *et al.*, 1944), which opened the door to molecular genetics.

1.1.3.2 Morphology, Growth Characteristics and Disease

Streptococcus pneumoniae are gram-positive cocci occurring in pairs or short chains. They are non-motile, spore forming and when freshly isolated are capsulate. They are aerobic or facultative aerobic fastidious organisms, growing best on media supplemented with 5% blood and 5% to 10% CO_2 at 37°C. When grown on blood agar *S. pneumoniae* produces a green halo of α-haemolysis around each colony. This typical appearance, a lack of catalase production, and a zone of inhibition around an optochin disk or solubility in bile salts are sufficient for presumptive identification of *S. pneumoniae* (Ross, 1996).

Pneumococci are involved chiefly in infections of the upper and lower respiratory tracts and are the most frequent bacterial cause of lobar and bronco-pneumonia in general practice and in hospital (Ross, 1996). Before the introduction of antibiotics pneumococcal pneumonia carried a mortality of 77% (Tilghman & Finland, 1937). The mortality rate decreased to 25% for bacteraemia pneumococcal disease between 1952 and 1962, after the introduction of antibiotic treatment and remained at a similar level of 28% from 1967 to 1970 (Austrian & Gold, 1964; Mufson *et al.*, 1974).

1.1.3.3 Virulence Factors

The pathogenicity of *S. pneumoniae* has been attributed to various structures, most of which are situated on its surface. The high morbidity caused by this microorganism

is still, however, poorly understood, and the list of virulence factors is probably far from complete. The disease manifestations are caused primarily by the host response to infection rather than the production of organism-specific toxin. The pathogenesis of *S. pneumoniae* infection is a complex interplay between pneumococcal virulence determinants and the host immune response.

The capsule has long been recognised as the major virulence factor of *S. pneumoniae*. Encapsulated strains were found to be at least 10^5 times more virulent than strains lacking the capsule (Avery & Dubos, 1931; Watson & Musher, 1990). The chemical structure of the capsular polysaccharides (PS) and to a lesser extent the thickness of the capsule determines the differential ability of serotypes to survive in the bloodstream and possibly to cause invasive disease. Capsular PS protect pneumococci from phagocytosis by polymorphonuclear leukocytes (Knecht *et al.*, 1970). The role of the capsule as a virulence factor is also well illustrated by the highly protective activity of anti-capsular antibody (AlonsoDeVelasco *et al.*, 1993). Tuomanen *et al* (1987) demonstrated that capsular PS were not necessary for inflammation but do contribute to the progression of the infection by inhibiting phagocytosis. There are currently 90 different serotypes.

i. Capsular serotypes

On the basis of differences in the capsular polysaccharide structure, *S. pneumoniae* may be divided into 90 different serotypes. Two types of nomenclature have been developed: one Danish and one American (AlonsoDeVelasco *et al.*, 1995). The Danish method classifies serotypes according to structural and antigenic

characteristics e.g. serotypes 6A and 6B differ only slightly from each other. The American nomenclature assigns the serotype number in the sequence of first isolation. Therefore, serotypes 6A and 6B are types 6 and 26 respectively in the American system. The Danish nomenclature is the most widely adopted. This system is based on the reactions to 48 antisera. Some antisera recognise specific serotypes whereas others recognise multiple serotypes within a serogroup e.g. serogroup 6 antisera recognises serotypes 6A and 6B. The isolates are first separated into groups and then each serogroup is further subdivided into the serotypes.

Capsular variability allows different serotypes to avoid immune detection by antibodies, which enables the organism to avoid phagocytosis and as such is an important virulence factor. Some serotypes are more associated with invasive disease than others. Serotypes 6, 14, 19 and 23 are the serogroups most often associated with serious infection in children (Robbins *et al.*, 1983). As small children often require antibiotic treatment for the disease, these serotypes are also often associated with antibiotic resistance (Dagan *et al.*, 1994; Butler *et al.*, 1995).

ii. Cell wall and cell wall polysaccharide

The *S. pneumoniae* cell wall (CW) is a dynamic structure composed of more than a dozen glycopeptides that are continuously inserted into and released from this circumferential, extracellular macromolecule (Tomasz, 1981; Garcia-Bustos *et al.*, 1987). In contrast to the capsular PS, purified peptidoglycan and especially CWPS have been found to induce inflammation similar to that seen after infection with the

whole pneumococci. Typical pneumococcal diseases such as otitis media, meningitis and pneumonia can be mimicked in animals that have been injected with purified CW or its degraded products (Tuomanen *et al.*, 1985; Tuomanen *et al.*, 1987; Carlsen *et al.*, 1992). The cell wall or more specifically the CWPS result in inflammatory effects due to activation of the alternative complement pathway (Winkelstein & Tomasz, 1977; Winkelstein & Tomasz, 1978). Interleukin-1 production is increased, which together with tumour necrosis factor, plays a pivotal role in the inflammation process (Riesenfeld-Orn *et al.*, 1989). The CW has also been shown to mediate the attachment of unencapsulated *S. pneumoniae* to endothelial cells (Geelen *et al.*, 1993). Enzymatic degradation of the cell wall releases components, which are more potent chemotactic factors that the intact CW. This finding is particularly relevant to the consequences of bacterial lysis due to antibiotic action (Tomasz & Saukkonen, 1989).

iii. Pneumococcal proteins

Various proteins have been suggested to be involved in the pathogenicity of *S. pneumoniae*. However, not all have been confirmed as virulence factors.

Pneumococcal surface protein A (PspA)

Antibody studies have demonstrated that PspA is located on the cell wall of *S. pneumoniae*. PspA has been confirmed as a pneumococcal virulence determinant. The function of PspA, a protective antigen for pneumococci, appears to be protection against host complement systems (Crain *et al.*, 1990; Yother & Briles, 1992; Yother

& White, 1994). Recent work has shown that PspA functions as a specific receptor for lactoferrin and hence plays an essential role in enabling iron acquisition by *S. pneumoniae* (Hammerschmidt *et al.*, 1999).

Adhesion proteins

Pneumococcal surface antigen A (PsaA) is a surface lipoprotein essential for *S. pneumoniae* virulence. The PsaA is a component of an ABC-type manganese permease membrane transport system and its likely function is to transport Mn^{2+} and Zn^{2+} into the cytoplasm of the bacteria. The *psa* operon appears to have a regulatory role in adhesion by affecting the expression of choline-binding proteins on the *S. pneumoniae* surface (Novak *et al.*, 1998).

Pneumococcal surface protein C (PspC), formerly known as choline-binding protein A (CbpA), is a surface protein that cross-reacts with PspA but unlike PspA is essential for pneumococcal carriage. It is thought to play an important role in the adhesion of pneumococci to human cells. It is also essential for pneumococci penetration of the blood brain barrier.

Neuraminidase is another virulence factor of *S. pneumoniae*, encoded by either the *nanA* or *nanB* genes, that is present on all strains of freshly isolated *S. pneumoniae* examined (O'Toole *et al.*, 1971; Berry *et al.*, 1996). This enzyme may facilitate attachment to the epithelial cells by cleaving sialic acid from the glycolipids and gangliosides found on the cell surface. Other proteins that may enhance the virulence

of pneumococci include hyaluronidase, neutrophil elastase inhibitor and peptide permeases.

iv. Pneumolysin

Pneumolysin is a cytoplasmic toxin released by autolysis of the cell. The toxin first binds to the target cell membrane and then forms a high molecular weight transmembrane pore (Bhakdi & Tranumjensen, 1986). This results in leakage of intracellular solutes and an influx of water resulting in lysis of the cell. Using this mechanism, pneumolysin is able to damage a wide range of eukaryotic cells, including bronchial epithelial cells, alveolar epithelial cell and pulmonary endothelium. The action on bronchial epithelium results in slowing of the ciliary beat, impairing the ability of the mucociliary escalator to clear particles effectively. Pneumolysin thus may enhance the inflammatory process. Pneumolysin also serves to facilitate bloodstream invasion by pneumococci and inhibits the respiratory bursts and chemotaxis of polymorphonuclear leukocytes (PMNC) (Paton & Ferrante, 1983).

v. Autolysins

Autolysins are members of a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacterial organisms. The action of these cell wall degrading enzymes ultimately leads to cell death. One of the direct implications is the release of cell wall components shown to be highly inflammatory in some animals. The indirect implication involves the release of cytoplasmic bacterial proteins including bacterial virulence factors such as pneumolysin (Berry *et al.*, 1989). The precise role of autolysin in *S. pneumoniae* virulence is still under debate.

1.1.3.4 Pathogenesis

Streptococcus pneumoniae is carried in the upper respiratory tract by many healthy individuals. The mechanisms used by *S. pneumoniae* to transfer from the nasopharynx to the lung or to the blood are poorly understood (Boulnois, 1992). Most infections do not occur after prolonged carriage but follow the acquisition of recently-acquired serotypes (Gray *et al.*, 1980; Boulnois, 1992). This suggests that the immune status of the host and the virulence of a strain both determine whether the pneumococci will remain in the nasopharynx or become invasive.

Failure of the specific and non-specific defences of the respiratory tract may facilitate access of pneumococci to the bronchi and lungs (Boulnois, 1992; Musher, 1992). Simultaneous damage of the epithelial layer by H_2O_2 (produced by pneumococci) and by pneumolysin may facilitate direct access to the blood. Epithelial damage by previous, probably viral, upper respiratory tract infections also increases the opportunity of pneumococci to reach the blood. From the blood the bacteria may migrate to the meninges or they may reach the meninges directly from the nasopharynx (Boulnois, 1992). Unrestrained multiplication of pneumococci in the lungs, meninges, or middle ear will result in pneumococci lysis with the release of cell wall products and pneumolysin. Pneumococcal lysis will in turn trigger the inflammatory response. There is increasing support for the hypothesis that such inflammation may be responsible for the morbidity and mortality caused by pneumococci infection (Musher, 1992).

Mosaic genes

In some species, such as streptococci, where genetic exchange occurs frequently, comparative studies of the structure of selected genes has shown that some strains contain parts of genes that are markedly different from those in other members of the species, such sequences have been referred to as mosaic genes. Mosaic genes result from occasional intragenic recombination between genetically distinct alleles. The resulting genes may express proteins with novel phenotypes, particularly if the newly incorporated DNA is from a different species or genus and so very different from the host DNA. Twenty to thirty percent base differences in the recombining genes are tolerated by RecA-mediated homologous recombination (Lorenz & Wackernagel, 1994).

In a population of bacteria most mosaic genes will be lost in a divergent population unless the mosaic gene expresses a phenotype, which confers a selective advantage to the bacteria and thus bacteria containing the mosaic gene would found a new population. The use of antibiotics has created a selective pressure, which selects bacteria with mosaic genes encoding proteins that provide resistance to the antibiotics, such as the ß-lactam antibiotics, the penicillins.

The penicillin-binding proteins (PBPs) in penicillin resistant *Neisseria* species and in *S. pneumoniae* represent some of the most extensively studied examples of mosaic genes. Penicillin binding proteins are the target proteins of the penicillin family of antimicrobial agents and intrinsic resistance (as opposed to ß-lactamase mediated

resistance) is a result of the reduced affinity of PBPs for penicillin. Four of the six pneumococcal PBPs have been shown to be phenotypically altered in resistant isolate, PBPs 1a, 2a, 2b and 2x (Laible *et al.*, 1991). Three of these PBPs have been characterised as being encoded by mosaic genes in resistant isolates, PBPs 1a, 2b and 2x. These PBPs had one or more blocks of sequence in which approximately 20% divergence had replaced homologous sequences of sensitive strains (Dowson *et al.*, 1989; Laible *et al.*, 1991; Martin *et al.*, 1992). The resistant sequences were interspersed with "sensitive sequences" that had less than 1% variation within one species (Smith *et al.*, 1991). Experiments have shown that in order to be penicillin resistant, *S. pneumoniae* must acquire low-affinity variants of the three PBPs encoded by mosaic genes, PBPs 1a, 2a and 2x. This initially seems unlikely, but nucleotide sequence data has shown that this has occurred (Dowson *et al.*, 1989; Dowson *et al.*, 1993; Kell *et al.*, 1993; Dowson *et al.*, 1994) and led to widespread penicillin resistance in *S. pneumoniae*.

No naturally occurring resistant streptococcal species exist that could account for the PBPs as resistance determinants for highly resistant *S. pneumoniae*. However, *pbp2x* genes from penicillin sensitive *Streptococcus oralis* and *Streptococcus mitis* were less than 4% divergent from the mosaic blocks in homologous genes of resistant *S. pneumoniae* (Dowson *et al.*, 1993; Sibold *et al.*, 1994). Also, the mosaic blocks of *pbp2b* genes in several resistant *S. pneumoniae* contained *S. mitis* DNA (Dowson *et al.*, 1993). Therefore, commensal streptococci could provide a reservoir of mosaic genes, which until they are integrated into *S. pneumoniae* do not result in the resistance phenotype.

1.1.3.5 Antibiotic resistance in *Streptococcus pneumoniae*

Pneumococcal resistance to antimicrobial agents originated in the beginning of the 20th century. Optochin-resistant pneumococci were identified in experimentally infected mice in 1912 and acquired optochin resistance during therapy of patients was documented in 1917. Clinical use of optochin was then limited by its severe side effects.

In 1939 resistance to the sulphonamide 2-sulphanilylaminopyridene was reported by the inability of the drug to cure experimentally-infected mice. The development of resistance during therapy was reported later that year in a human case of pneumococcal meningitis (Ross, 1939). Sulphadiazine resistance during therapy and spread of the resistant strain to a second patient was reported in 1943 (Frisch *et al.*, 1943).

Laboratory derived penicillin-resistant *S. pneumoniae* were developed in the 1940s (McKee & Houck, 1943) but it was not until 1965 that the first clinical isolate with reduced penicillin susceptibility was discovered in Boston (Kislak *et al.*, 1965) and the first penicillin-resistant clinical strain isolated in Australia reported in 1967 (Hansman & Bullen, 1967). This then spread to New Guinea where 12% of 518 New Guinean isolates were penicillin-resistant (Hansman *et al.*, 1974). From 1974 to 1980, the reported distribution of penicillin-resistant strains became worldwide. In the UK, resistance increased from 0.1% in 1977 (Howard *et al.*, 1978) to 4% in 1987 (Nair, 1988) and 9.1% in 1997/1998 (Felmingham *et al.*, 2000). The number of

penicillin-resistant *S. pneumoniae* is still increasing even though we have been aware of clinical penicillin-resistant *S. pneumoniae* for 35 years.

Trimethoprim/Sulphamethoxazole (TMP-SMZ) resistance was identified in *S. pneumoniae* for the first time in 1972 (Howe & Wilson, 1972). Trimethoprim/Sulphamethoxazole resistance is associated with multiple resistant *S. pneumoniae* isolated both from children in hospital and healthy children in the community (Jacobs *et al.*, 1978; Klugman *et al.*, 1986).

Tetracycline was widely used in the 1960s for the management of acute exacerbations of chronic bronchitis. The first case of tetracycline-resistant *S. pneumoniae* was reported in Australia in 1963 (Evans & Hansman, 1963) and also in the UK due to an outbreak of 10 resistant strains in Liverpool (Turner, 1963). Tetracycline resistance increased to 23% in the Liverpool Royal Infirmary by 1968 (Percival *et al.*, 1969). However, pneumococcal resistance to tetracycline declined after 1969 most likely due to the reduction in tetracycline prescription (Howard *et al.*, 1978).

Fluoroquinolone resistance surveillance in *S. pneumoniae* is a relatively recent occurrence. The levels of resistance are extremely low worldwide. In 1996/1997 the highest level of pneumococci resistance to ciprofloxacin (≥1mg/L) or ofloxacin (≥1mg/L) worldwide was 0.5%. The combined resistances of ciprofloxacin and ofloxacin were 0.1% and 0.04% for 1996 and 1997 respectively (Felmingham & Grüneberg, 2000). Between 1997 and 1999 S. *pneumoniae* resistant to levofloxacin

or gatifloxacin was ≥1% for both fluoroquinolones (Hoban *et al.*, 2001b). While the levels of fluoroquinolone resistance were extremely low we must exercise caution so that more resistant strains do not emerge in the future.

Multiple resistant *S. pneumoniae* first emerged in 1977 in South Africa (Jacobs *et al.*, 1978). This isolate was penicillin, tetracycline, erythromycin, clindamycin, chloramphenicol, and TMP-SMZ resistant. While the number of isolates with multiple resistances is low in comparison to resistance to one antimicrobial agent, multiple resistances are increasing in many countries. In the UK ten years after the first multiple resistant strain was identified, 40 multiple resistant isolates were reported (George *et al.*, 1987). Today, isolation of multiply resistant *S. pneumoniae* from both adults and children has been reported around the world.

The first report of erythromycin-resistant pneumococci, in 1964, was of 6 clinical strains with erythromycin MICs of ≥5 mg/L. However, it was Dixon in 1967 who brought this finding to the medical communities attention in a letter to *The Lancet* (Dixon, 1967). During the 1970s and 1980s no erythromycin-resistant isolates were found in studies conducted in the UK (Howard *et al.*, 1978), Hong Kong (Ling *et al.*, 1983) or Germany (Federal Republic) (Kaufhold *et al.*, 1987). The current situation for erythromycin-resistant strains in these countries is UK 13.2%, Hong Kong 72.9% and Germany 15.7% (Felmingham, 2002). In the last 20 years the situation of erythromycin-resistant *S. pneumoniae* has become critical in some countries and resistance is ever increasing. The idea that resistance may increase in countries where the drug was widely used evolved during the late 1980s due to the identification of a

high prevalence of erythromycin-resistant strains, associated with multiple resistance, in South Africa (Felmingham, 2002).

1.2 Macrolide antimicrobial agents

The first macrolide to be used clinically was erythromycin A in the 1950s. At this time erythromycin was a valuable novel drug, which could be used against emerging penicillin resistant *S. pneumoniae* and could also treat patients with ß-lactam intolerance. Erythromycin is a natural antibiotic isolated from *Saccharopolyspora erythrea* formerly *Streptomyces erythreus*. It consists of a 14-membered lactone ring with 2 attached sugar moieties at C3 and C5 as shown in figure 1.

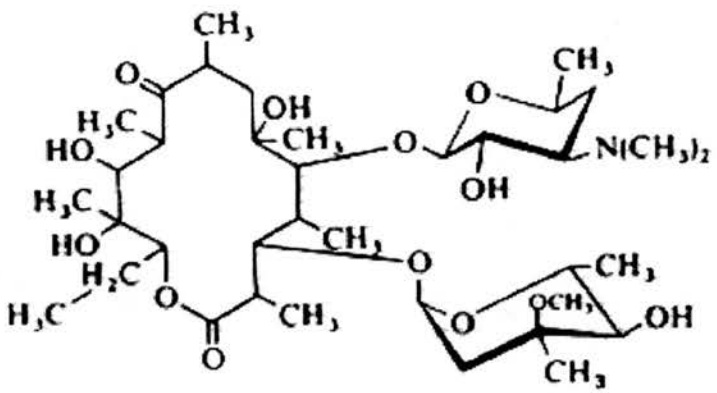


Figure 1. Chemical structure of Erythromycin A.

Erythromycin activity is primarily against gram-positive bacteria but also has activity against atypical pathogens such as *Mycoplasma pneumoniae*, *Legionella pneumophilla* and *C. pneumoniae*. Macrolides are mainly bacteriostatic agents but bactericidal activity may be achieved at high concentrations. They bind to the 23S rRNA in the 50S-subunit of the prokaryotic ribosomes and prevent protein synthesis (Brisson-Noël *et al.*, 1988). Macrolides belong to a chemically distinct but

functionally overlapping family of antimicrobial agents, which are all protein synthesis inhibitors. This group consists of macrolides (M), lincosamides (L), streptogramin B (S_B) antimicrobial agents and recently ketolides (K). Although it has been extensively studied since the 1960s the exact mechanism of action of the macrolides has not yet been fully elucidated. However, different theories exist on the way in which macrolides inhibit protein synthesis.

One theory suggests that macrolides act by blocking peptide elongation. During elongation the peptide chain grows through amino acid addition, which is catalysed by peptidyl transferase. The peptide chain is translocated from the aminoacyl acceptor (A) site to the peptidyl (P) donor site (see figure 2). Erythromycin is thought to block translocation of the peptidyl-transfer RNA (tRNA), which is the tRNA bearing the nascent peptide, or inhibit peptidyl transferase thus causing peptidyl-tRNA translocation to cease (Monro & Vazquez, 1967; Brisson-Noël *et al.*, 1988; Mazzei *et al.*, 1993).

Another proposed theory is that macrolides inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. The weakening of the bonds between the ribosomes and the peptidyl-tRNA during translocation causes protein synthesis inhibition. The hypothesis is that more peptidyl-tRNA is produced as a result of repeated ribosome peptidyl-tRNA dissociation with the accumulation of dissociated peptidyl-tRNA leading to inhibition of protein synthesis and cell death. Menninger (1985) suggested that the blocking of protein synthesis by erythromycin

would occur after dissociation of peptidyl-tRNA due to its accumulation (Menninger & Otto, 1982; Menninger, 1985; Brisson-Noël *et al.*,1988; Mazzei *et al.*,1993).

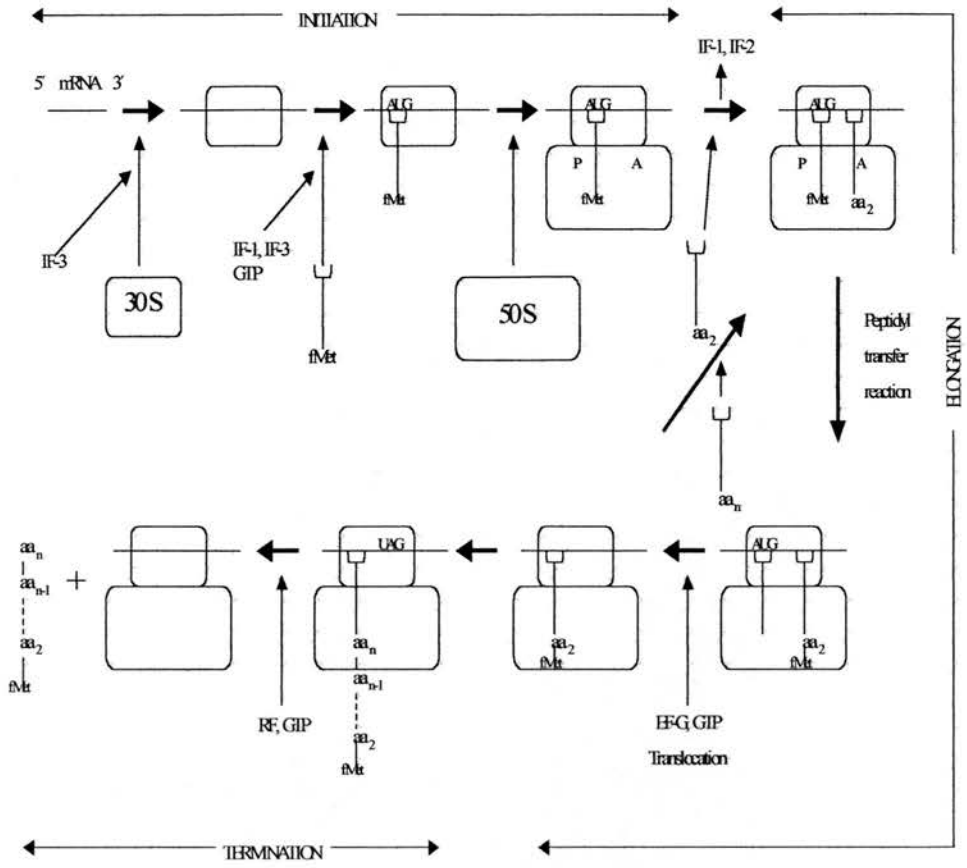


Figure 2. Simplified scheme of protein synthesis mechanism (adapted from Brisson-Noël *et al.*, 1988). A, aminoacyl acceptor site; aa, amino acid; EF, elongation factor; fMet, formylmethionine; P, peptidyl donor site; RF, releasing factor.

The most recent theory proposed by Champney and Burdine (1995) postulated that macrolides might secondarily inhibit 50S ribosomal subunit assembly. Erythromycin

causes the accumulation of a 50S subunit precursor particle in cells. This binds erythromycin and the stalled 50S subunit particle, containing 23S rRNA and 5S rRNA, is then degraded by cellular ribonucleases. These results suggest a second target for macrolides and that both the elongation step of polypeptide chain formation and the initial steps of 50S subunit assembly are affected by these compounds (Champney & Burdine, 1995).

Resistance to erythromycin emerged quite rapidly after its clinical introduction (Weisblum, 1995a). In order to overcome the problem of erythromycin resistance, a number of semi-synthetic derivatives such as clarithromycin and azithromycin have been developed. These macrolides have better pharmacokinetic profiles than erythromycin, induce fewer gastro-intestinal side effects and offer improved activity against *H. influenzae* and atypical pathogens (Neu, 1991; Zuckerman, 2000). However, resistance to the new macrolides, increased erythromycin resistance and MLS_B resistance have led again to a search for new agents with activity against such resistant bacteria and also low potential to select for or induce resistance and cross-resistance. The ketolides, of which telithromycin is the first in clinical use, represent the new generation of antimicrobial agents to tackle these problems.

1.3 Telithromycin

Telithromycin is a semi synthetic 14-membered ring macrolide derivative as shown in figure 3. There are three structural advantages to telithromycin not present in the macrolides.

1. Telithromycin contains a 3-keto function instead of an L-cladinose moiety, a neutral sugar long thought to be essential for antibacterial activity, of the macrolides. This substitution enables telithromycin activity against erythromycin resistant strains (Bonnefoy *et al.*, 1997).
2. The hydroxyl group at C6 of erythromycin, is replaced by a methoxy group. The methoxy group and the keto group together give telithromycin excellent acid and gastrointestinal stability compared with the macrolides (Bryskier, 1998).
3. The carbamate C11-C12 extension gives telithromycin improved binding affinity to the ribosome and is partly responsible for the activity of telithromycin against MLS_B-resistant ribosomes compared to the macrolides (Hansen *et al.*, 1999; Douthwaite *et al.*, 2000).

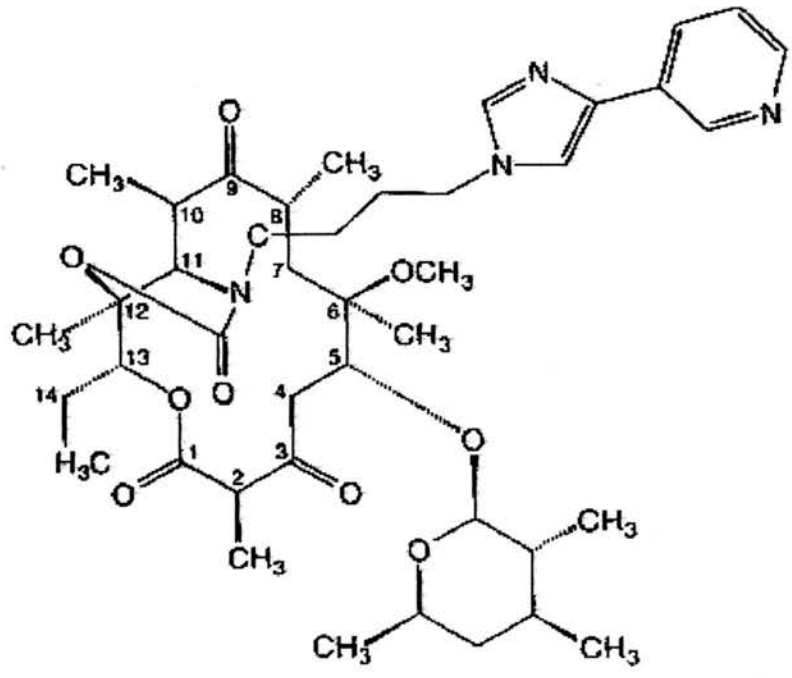


Figure 3. Chemical structure of telithromycin.

The main target bacteria of telithromycin are the macrolide resistant and MLS_B resistant *S. pneumoniae*. Telithromycin has excellent activity against macrolide susceptible *S. pneumoniae* (Barry *et al.*, 1998; Pankuch *et al.*, 1998; Davies *et al.*, 2000a) and retains good activity against penicillin resistant (Barry *et al.*, 1998; Pankuch *et al.*, 1998; Hoban *et al.*, 1999) and erythromycin resistant strains (Barry *et al.*, 1998; Pankuch *et al.*, 1998; Hoban *et al.*, 1999). Telithromycin has been reported to have bactericidal activity against *S. pneumoniae* (Hamilton-Miller & Shah, 1998), but is generally thought to be a bacteriostatic drug. Telithromycin has similar activity to the macrolides against gram-negative bacteria (Biedenbach *et al.*, 1998; Wootton *et al.*, 1999) and atypical respiratory tract pathogens (Roblin & Hammerschlag, 1998; Bebear *et al.*, 2000).

1.3.1 Mode of Action

Ketolides inhibit bacterial protein synthesis in a very similar manner to the macrolides but with greater effect (Hansen *et al.*, 1999; Douthwaite *et al.*, 2000). Macrolides and ketolides bind to the same region of the 23S rRNA of the assembled 50S subunit but the nature and strength of binding differ. Erythromycin, clarithromycin, azithromycin and telithromycin interact with domain II and V of the 23S rRNA according to foot printing experiments (Xiong *et al* 1999; Douthwaite *et al* 2000). The domain V region of interaction of the 23S rRNA with the macrolides and ketolides is known as the peptidyl-transferase region.

As amino acids are added together to form a nascent peptide the chain passes through a "peptide exit tunnel" in the 50S ribosomal subunit close to the peptidyl transferase region. The MLS_B antimicrobial agents and telithromycin bind to the 23S rRNA and so block the peptide exit channel. The MLS_B antimicrobial agents and telithromycin all protect nucleotide residues Adenine (A) 2058, A2059 and Guanine (G) 2505, which means that they all bind to each of these sites in domain V of the 23S rRNA. Telithromycin also protects A752 in domain II whereas the L-cladinose of erythromycin and clarithromycin enhance accessibility to it and thus do not bind to it. This extra binding site affinity is also associated with the C11-C12 carbamate link present in telithromycin but not erythromycin or clarithromycin. The relative positions of domains II and V in the 23S rRNA are such that the distance between the nucleotides binding telithromycin in domain V are close enough to the A752 in

domain II to be spanned by the carbon11-carbon12 carbamate link of telithromycin (Hansen *et al.*, 1999; Xiong *et al.*, 1999), as indicated in figure 4.

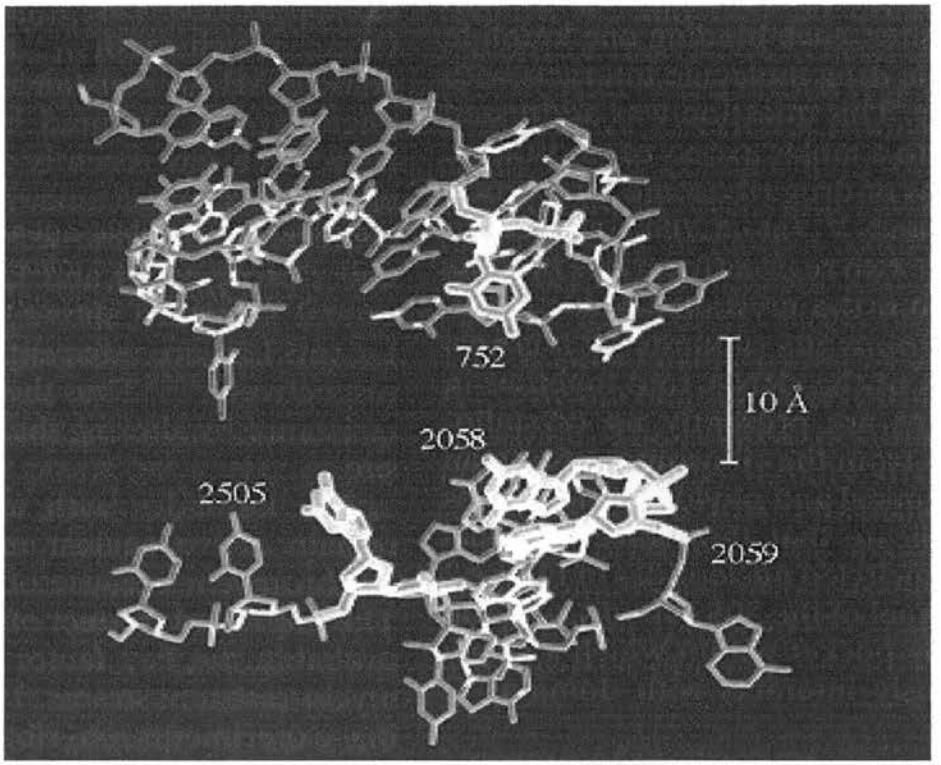


Figure 4. Tertiary structure of 23S rRNA domains II and V with spanning from Douthwaite *et al.*, (2001).

Telithromycin was found to bind approximately 10-fold tighter to the ribosome than erythromycin. This is thought to be due mainly to the binding of the C11-C12 link to domain II. In fact, addition of a C11-C12 carbamate to clarithromycin increased binding affinity approximately 5-fold. The two regions domain II and domain V form one binding site for telithromycin not two distinct binding sites (Hansen *et al.*, 1999; Douthwaite *et al.*, 2000). As telithromycin has improved binding capacity and an additional binding site or sites, compared to erythromycin it follows that it has improved activity and also activity against macrolide resistant strains.

1.4 Mechanisms of Macrolide Resistance

The two main mechanisms of macrolide resistance in *S. pneumoniae* are target modification and efflux. A methyl-transferase gene mediates target modification: *ermB* (erythromycin-resistance methylase). The *ermB* gene has been located on transposons and plasmids in both gram positive and gram-negative bacteria. In pneumococci, the gene is borne only by conjugative transposons related to Tn*1545*, Tn*1545*-like elements, or a Tn*917*-like element that is part of a larger composite transposon, Tn*3872* (McDougal *et al.*, 1998; Trieu-Cuot *et al.*, 1990). The target modification causes resistance not only to macrolides but also to lincosamides and streptogramin B antibiotics and is associated with high-level macrolide resistance (Weisblum, 1995a).

erm genes cause methylation of the binding site of the MLS_B antibiotics within the peptidyl transferase centre of the 23S rRNA. In *S. pneumoniae* the *ermB* gene mediates methylation although recently an *ermA* gene has been also associated with macrolide resistant *S. pneumoniae* (Syrogiannopoulos *et al.*, 2001; Nagai *et al.*, 2002). Methylation by the *erm* associated methylase occurs at the adenine at position 2058 (Arthur *et al.*, 1987). Methylation can occur as monomethylation or dimethylation. It has recently been shown that variations in mono- or dimethylation lead to different resistance phenotypes (Liu & Douthwaite, 2002). The monomethylation of A2058 resulted in intermediate resistance to erythromycin and clarithromycin, high-level resistance to lincosamides but no resistance to the ketolides. Dimethylation conferred high-level resistance to macrolides, lincosamides

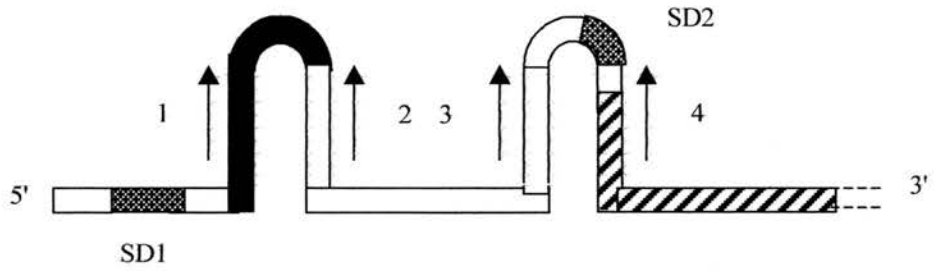
and resistance to the ketolides. ErmB and ErmA(TR) are both A2058 dimethyltransferases. This however, is not reflected in the MICs of *S. pneumoniae* to date. One of the reasons for this may be in the way in which the ErmB and ErmA methylate the A2058. Due to metabolic conditions, such as the availability of S-adenosylmethionine from which the methyl group is removed, ErmB could only monomethylate the A2058. Thus *S. pneumoniae* containing a mixture of monomethylated and dimethylated ribosomes could have macrolide resistant but ketolide sensitive phenotypes (Liu & Douthwaite, 2002). This theory does require more evidence as little is currently known about this process in *S. pneumoniae*. The addition of the methyl group(s) causes conformational changes in the 50S ribosomal subunit, which prevents the MLS_B antimicrobial agents from binding to their site of interaction with the 23S rRNA and so prevents them from inhibiting protein synthesis. Expression of resistance due to *erm* methylation can be inducible or consititutive.

1.4.1 Inducible resistance

The inducible expression of MLS_B resistance is putatively controlled at a post-transcriptional level by a regulatory region upstream from the *erm* gene (Weisblum, 1995b). The regulation is not related to the class of *erm* gene. When expression is inducible, *ermB* mRNA is synthesised but in an inactive conformation I, figure 5, and becomes active only in the presence of inducing macrolides (conformation II in figure 5). In the non-induced state the conformation of the control peptide upstream from the *erm* gene is such that the ribosome-binding site needed for transcription of

the *erm* gene is not accessible to the ribosome. Only the sequence corresponding to the control peptide is translated (see figure 5 conformation I). When present, erythromycin binds to the ribosomes, including those involved in the synthesis of the control peptide, and causes them to stall. This stalling is thought to cause displacement of the stem-loop structure by conformational rearrangements (see figure 5 conformation II). The ribosome-binding site is then available to the ribosome and so initiation of translation of the methylase may occur. When all of the ribosomes are methylated stalling does not occur and so the mRNA returns to its original conformation. High-level cross-resistance to MLS_B antimicrobial agents in clinical streptococci is mainly due to inducible expression of resistance (Leclercq & Courvalin, 1991).

Conformation I



Conformation II

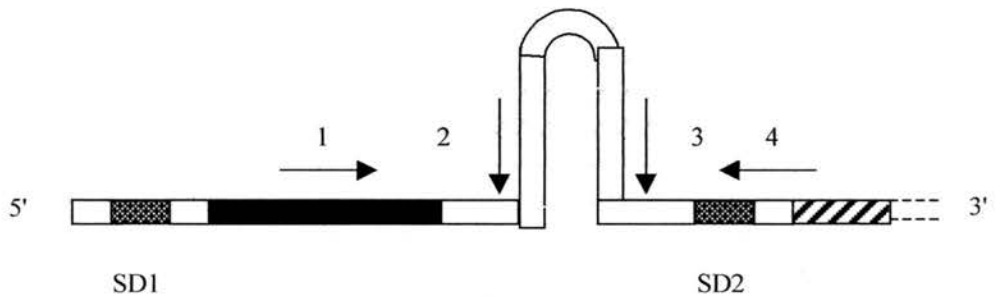


Figure 5. Alternative conformations of the mRNA from the inducible *ermC* gene from pNE194. Shown are the secondary structures of the mRNA in the absence (conformation I) or in the presence (conformation II) of erythromycin. Symbols: solid white is the mRNA, solid black is the sequence of the control peptide, diagonal black lines are the sequence of the methylase, 1, 2, 3 and 4 represent inverted repeats. SD1 and SD2 are the ribosome binding sites 1 and 2.

1.4.2 Constitutive resistance

When expression is consititutive, the *ermB* mRNA is active (conformation II in figure 5). The exact mechanism or mechanisms of constitutive resistance have not been fully elucidated in *S. pneumoniae*. Constitutive expression of MLS_B resistance in various bacteria has been associated with deletions or mutations in the regulatory region upstream from the *erm* gene. Rosato *et al* (1998) showed a deletion of most of the regulatory region was responsible for constitutive expression of the *erm* gene from *Streptococcus agalactiae*. Constitutive expression in clinical isolates of *S. pneumoniae* has been associated with a deletion of the control peptide and one ribosome-binding site upstream from the *ermB* gene and also mutations within the control peptide associated with constitutive resistance in another strain. One strain, which had constitutive MLS_B resistance was, however, without deletion or mutation in this region and was identical to that of the inducible strains. Therefore, modifications other than those in the *erm* upstream region are also possibly responsible for constitutive resistance (Rosato *et al.*, 1999). Two *Streptococcus pneumoniae* clinical isolates with deletions in the leader peptides of their *ermB* genes have been isolated, one of which was highly ketolide resistant. In these strains however there were also other mutations, both had 3 amino acid mutations in the ErmB protein itself and the ketolide resistant strain also had mutations in the L4 riboprotein (Tait-Kamradt *et al.*, 2001).

1.4.3 Macrolide efflux

It is only recently that another mechanism, other than methylation, has been identified. Before 1996 efflux of macrolides had not been specifically described in streptococci even though in 1993, Seppälä *et al* (1993) described *Streptococcus pyogenes* strains that were erythromycin resistant and clindamycin susceptible, known as the MS phenotype. Sutcliffe *et al* in 1996 described, for the first time, an efflux mechanism in both *S. pneumoniae* and *S. pyogenes*. They noticed that there was no change in binding of macrolides to the ribosomes, indicating no change in binding site of the macrolides. The efflux pump was distinguishable from the efflux system in staphylococci also bearing the MS phenotype. Efflux pumps do not modify either the antimicrobial agent or the antibiotic target, but instead pump the antibiotic out of the cell, keeping intracellular concentrations low and ribosomes free from antibiotic.

The macrolide efflux gene, *mefE*, was detected in *S. pneumoniae* in 1997 (Tait-Kamradt *et al.*, 1997). The gene was found to have 90% homology to the *mefA* gene of *S. pyogenes*. Both have since been put into the same class of macrolide efflux genes. In *S. pyogenes* strains with an M phenotype the *mefA* encodes a 44.2 KDa protein with homology at amino acid level to other efflux proteins. The protein encoded by *mefE* in *S. pneumoniae* is also a hydrophobic protein with homologies to other transporters or efflux proteins. *Streptococcus pneumoniae* strains with *mefE* genes are resistant to 14- and 15-membered macrolides but clindamycin and

streptogramin B sensitive. The macrolide resistance is of a lower level than those with either an *ermB* gene or ribosomal changes.

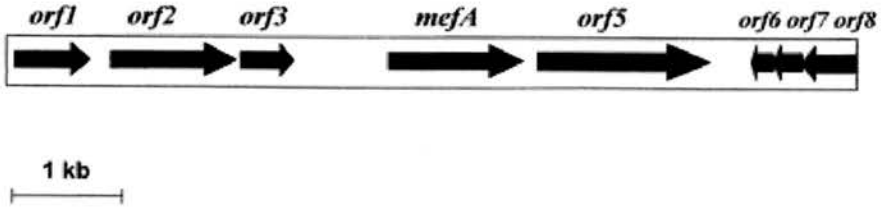


Figure 6a. *mefA* chromosomal element *Tn1207.1.*

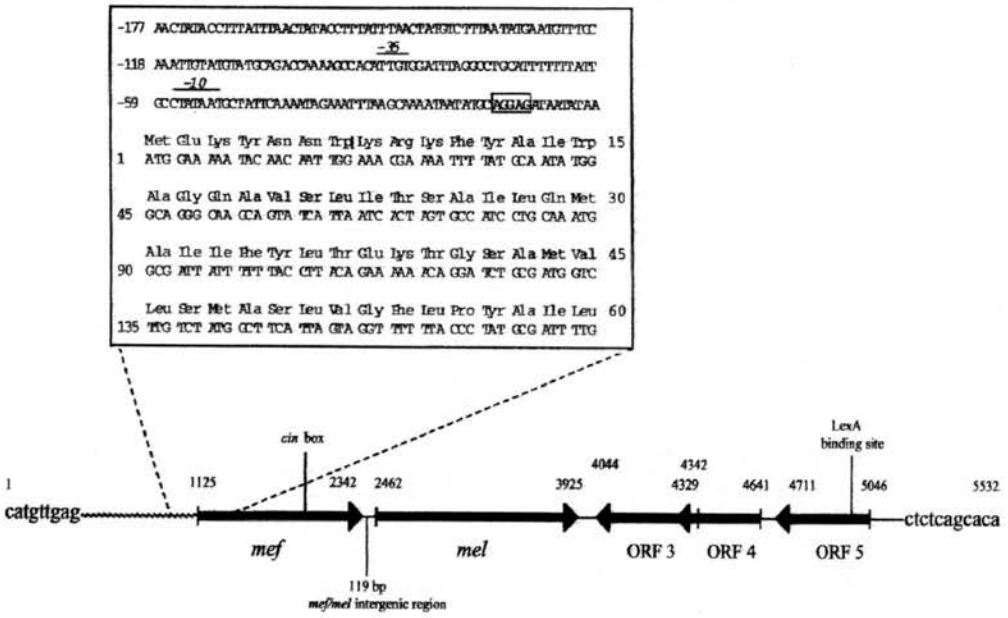


Figure 6b. The *mefE* genetic element *mega.*

The *mefA* genetic element was first described in 2000 (Santagati *et al.*, 2000) as a chromosomal element designated Tn*1207.1*, 7244 base pairs in size as shown in figure 6a. The entire element consisted of 8 open-reading frames (orf) with the *mef* gene between orfs 3 and 5. Adjacent to the *mef* gene was an orf homologous to *msr(SA)* and *vga(A). msr(SA)* confers resistance to macrolides and streptogramin B

and *vga(A)* confers resistance to streptogramin A antibiotics in *Staphylococcus aureus* (Roberts *et al.*, 1999). Gay and Stephens (2001) described a 5.4 or 5.5 Kb genetic element containing a *mefE* gene called *mega*, shown in figure 6b. This had similarities and differences to Tn*1207.1*. The first 4 orfs of *mega* and orfs 4 to 7 of Tn*1207.1* had >94% identity at nucleotide level. In *mega* the orf sequence 3' of the *mefE* gene was designated *mel*. The *mel* orf is also a homologue of *msrA* in staphylococci, which encodes an ATP-binding cassette to provide the energy for efflux. The *mefE* and *mel* are co-transcribed, which suggests that both are required for efflux. One important difference between *mefA* and *mefE* is their site insertion into the chromosome. The *mefA* element was found to be integrated at a single site in the chromosome whereas *mefE* was inserted at different chromosomal locations. In light of this information it may be wise to refer to the *mef* genes seperately as *mefA* and *mefE*.

Resistance in *mef* positive strains is stable. However, resistance to macrolides was found not to be stable in mutants derived from parent strains containing *mef* genes. The macrolide and telithromycin MICs reverted to the parent MIC after 10 passages on antibiotic-free medium (Davies *et al.*, 2000b).

The efflux *mef* mediated system is a current threat to 14- and 15-membered macrolides, but a *mef* mediated macrolide resistant *S. pneumoniae* infection would be the easiest macrolide resistant infection to treat as it could still be treated with lincosamides and streptogramin B or ketolides. This is currently the situation but

there is always potential for the efflux pump to change and also pump these antimicrobial agents out of the cell.

1.4.4 Macrolide resistance worldwide patterns

ermB and *mef* genes are disseminated throughout the world in *S. pneumoniae*. However, there is an uneven distribution of each gene. In the US and Canada surveillance studies revealed that the macrolide resistant (M) phenotype associated with the *mef* gene and efflux predominates. In Europe and the Asia-Pacific rim, the majority of macrolide resistant *S. pneumoniae* had an MLS_B resistance phenotype linked to the *ermB* gene (Hoban *et al.*, 2001b). This variation in distribution could be related to different antibiotic consumption and differential selective pressures or the clonal spread of erythromycin resistant strains or determinants within specific geographical areas.

1.4.5 *mef* and *erm* genes

While the resistance genes *ermB* and *mef* are usually isolated alone, there have been reports from South Africa (McGee *et al.*, 2001), Japan (Nishijima *et al.*, 1999), France (Marchandin *et al.*, 2001), Spain (Morosini *et al.*, 2001), Great Britain and Ireland (Farrell *et al.*, 2001) and Canada (Hoban *et al.*, 2001a) of *S. pneumoniae* strains with both genes present. The *ermB-mef* positive strains identified to date have all been clinical isolates. The percentage of *ermB-mef* positive strains is low in comparison to those containing only 1 of the genes.

The largest number of *S. pneumoniae* isolated in one study with both genes was in South Africa where a total of 36 strains were isolated from 4 different cities in 1999. All strains showed high-level resistance to erythromycin and clindamycin which is associated with the *ermB* gene, but in addition these strains also showed high level penicillin resistance, as well as resistance to chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole. Using BOX-PCR and pulsed-field gel electrophoresis (PFGE), 30 of the 36 isolates were shown to belong to a single multiply resistant clone (McGee *et al.*, 2001). The relatedness of this clone to the *ermB-mef* positive strains from other countries remains to be determined.

The *ermB-mef* isolates from around the world all showed high level resistance to erythromycin and clindamycin, which is the same resistance pattern as strains harbouring only the *ermB* gene. Therefore, it appears that the *ermB* gene is predominately expressed over the *mef* gene. Phenotypic tests alone are therefore not sufficient to investigate the presence of the *mef* gene when it exists in association with the *ermB* gene. The MIC of telithromycin against 6 of the *ermB-mef* positive strains tested was ≤0.002 to 0.06 mg/L (Hoban *et al.*, 2001a). Due to the low number of strains it is impossible to extrapolate much information from these results, except that telithromycin had good activity against these 7 strains.

1.4.6 Ribosomal mutations

Macrolide resistance in clinical isolates and laboratory-derived strains of *S. pneumoniae* have been linked to alterations of specific nucleotides in the 23S rRNA of the ribosomal subunit, described in figure 7. Mutations at A2058 are the most frequently identified ribosomal mutations associated with macrolide resistance in 23S rRNA (Vester & Douthwaite, 2001). Specifically in *S. pneumoniae* mutations have been located at nucleotides 2058, 2059, 2062 and 2611 (Tait-Kamradt *et al.*, 2000a; Depardieu & Courvalin, 2001; Farrell *et al.*, 2002; Pihlajamäki *et al.*, 2002). These ribosomal mutations were identified in clinical isolates, which did not contain the *ermB* or *mefA* genes. In addition to these mutation sites a mutation in the hairpin 35 region of the domain II of the 23S rRNA has been associated with macrolide and ketolide resistance in a laboratory derived mutant of *S. pneumoniae* (Canu *et al.*, 2002).

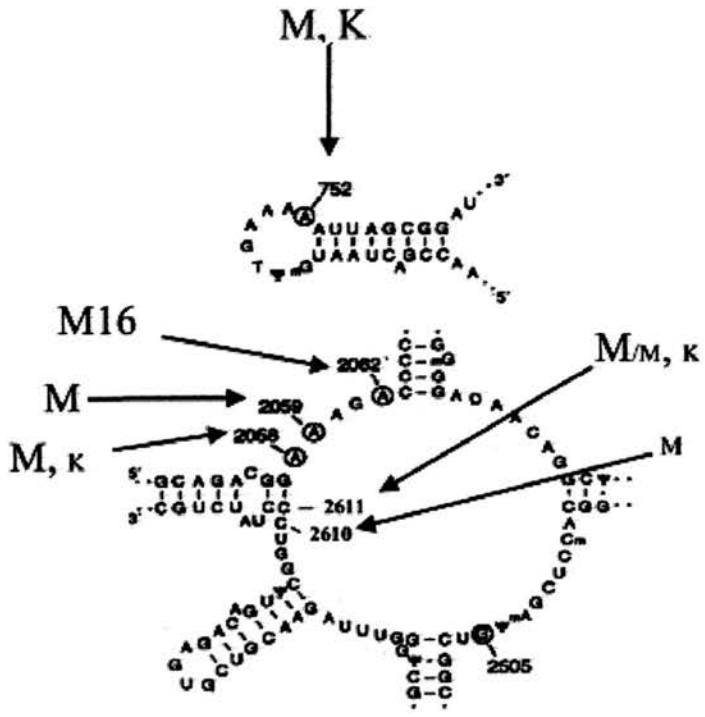


Figure 7. Secondary structure of domain V of 23S rRNA in *E. coli* (adapted from Leclercq & Courvalin, 2002). Nucleotides which are protected by erthromycin are circled. Arrows indicate mutations conferring macrolide resistance on *S. pneumoniae*. Capital letters correspond to the antibiotic groups; M, macrolides; M16, 16-membered macrolides; K, ketolides. Small capital letters denote low-level resistance.

Streptococcus pneumoniae has 4 copies of the 23S rRNA gene. The level of resistance depends not only on the type or position of mutation but also the number of copies containing the mutation. A laboratory-derived mutant with A2058G changes in 2 copies of the gene resulted in high-level macrolide resistance (Tait-Kamradt *et al.*, 2000b). In clinical isolates no such mutant has so far been isolated, however, 3 isolates with A2058G mutations in 3 of the 4 copies were highly macrolide resistant (Farrell *et al.*, 2002). Adenine to uracil (U) mutations in 3 copies of the genes at position 2058 also conferred macrolide resistance *in vitro* (Canu *et al.*, 2002). Both A2059G (Tait-Kamradt *et al.*, 2000a; Farrell *et al.*, 2002) and

A2059C (Pihlajamäki *et al.*, 2002) have been identified in clinical isolates. The A2059G mutation has also been created in laboratory-derived mutants (Tait-Kamradt *et al.*, 2000b). Clinical strains with the A2059G in 1, 2, 3 or 4 copies of the 23S rRNA have been isolated. It appears however, that the A2059G mutations in at least 2 copies of the genes are required to confer macrolide resistance. The A2059C mutation in 2, 3 or 4 copies resulted in the same high-level macrolide resistance (Pihlajamäki *et al.*, 2002).

The A2062C mutation has only been identified in 1 clinical isolate of *S. pneumoniae*, which was in all 4 copies of the 23S rRNA genes (Depardieu & Courvalin 2001). This set of mutations lead to resistance to 16-membered macrolides and streptogramins only. A mutation from cytosine to guanine at nucleotide 2611 resulted in macrolide resistance in clinical strains with 3 or 4 mutated alleles (Farrell *et al.*, 2002; Pihlajamäki *et al.*, 2002) and also laboratory-derived mutants with 4 altered alleles (Tait-Kamradt *et al.*, 2000b). However, laboratory mutants with cytosine to adenine or uracil changes were macrolide sensitive (Tait-Kamradt *et al.*, 2000b; Canu *et al.*, 2002). Only laboratory-derived mutants with a C2610U mutation have so far been identified but these were macrolide sensitive (Canu *et al.*, 2002).

One laboratory derived *S. pneumoniae* strain was found to have a 1 adenine deletion in the series of 4 located at positions 749 to 752 in the hairpin 35 of domain II (Canu *et al.*, 2002). The MIC of macrolides increased between 250 to 1000-fold to greater than 32mg/L and the MIC of telithromycin also increased 500-fold to an MIC of 4mg/L. This finding is consistent with previous reports of a single point mutation,

U754A, in a laboratory strain of *E. coli*, which was sufficient to render the cells resistant to telithromycin (Xiong *et al.*, 1999). It also confirms the importance of domain II, hairpin 35 in telithromycin binding.

Although reports of mutations of the 23S rRNA genes associated with macrolide resistance in clinical isolates of *S. pneumoniae* are low this does not make it any less important. Recently, the first report of azithromycin treatment failure due to an A2059G mutation was published. The patient was a 53-year-old woman with no history of smoking or alcohol abuse (Kays *et al.*, 2002).

The mechanism behind the 23S rRNA mutations causing resistance is thought to be the prevention of MLS_B antimicrobial agents binding to their site of ribosome interaction. The deletion or alteration of the domain II binding site confers the same fate to the ketolide telithromycin. Even though mutations in the domain V of the 23S rRNA have not been linked to telithromycin resistance in *S. pneumoniae*, laboratory derived mutations have shown an increase in telithromycin MIC due to the alterations (Tait-Kamradt *et al.*, 2000b; Canu *et al.*, 2002). The A2058G mutation in 2 or 3 copies of the genes increased the telithromycin MIC 10-fold and 16-fold respectively. The A2059G mutation in 2 and 4 copies of the 23S rRNA genes caused 3-fold and 4-fold increases in telithromycin MICs respectively. The mutation at nucleotide 2611 from cytosine to guanine in all 4 alleles, *in vitro*, caused the highest increase of a 130-fold increase in telithromycin MIC *in vitro*. Other mutations at this nucleotide from cytosine to adenine or uracil resulted in less than 10-fold increases in telithromycin MIC. These *in vitro* experiments suggest that the C2611G mutation is

the most potent mutation in domain V to date against telithromycin and thus is involved in telithromycin binding. Only a few studies have been carried out to date on telithromycin resistance and drug binding and as such no definite conclusions regarding binding sites can be made.

The ribosome consists not only of rRNA but also riboproteins, which interact with the rRNA to form the ribosomal subunits of the ribosome. Two such riboproteins are L4 and L22. Mutations in both of these proteins have been implicated in macrolide resistance. Mutations conferring resistance to erythromycin were first identified in ribosomal proteins L22 and L4 of *E. coli* (Wittmann *et al.*, 1973). Macrolide resistance mutations have also been located in a *Bacillus subtilis* (Tipper *et al.*, 1977) homologue of the *E. coli* L22 and in the L4 of *Bacillus stearothermophilus* (Sharrock *et al.*, 1981; Schnier *et al.*, 1990). Recently, mutations in both of these riboproteins have been associated with macrolide resistance in *S. pneumoniae*.

Riboprotein L4 is a 207 amino acid protein and L22 a 114 amino acid protein in *S. pneumoniae* (Tettelin *et al.*, 2001). In the assembly of the 50s ribosomal subunit L4 and L22 bind directly to the domain I region of the 23S rRNA. The primary binding sites of L4 and L22 are not yet fully elucidated. Positively-charged residues of both L4 and L22 interact with the negatively-charged phosphate groups of the RNA forming nucleic-acid-protein complexes (Unge *et al.*, 1998; Worbs *et al.*, 2000). Erythromycin resistance mutation studies of these riboproteins implied that they also have interactions with the central loop of domain V of the rRNA. Gregory and Dahlberg (1999), also showed that these proteins have multiple contacts with

rRNA in domains II, III and V of the rRNA. L22 binding is strongly dependent on L4.

In protein formation by the ribosome, the nascent peptide chain exits the ribosome via a tunnel. This tunnel is fenced by long extensions of L4 and L22 riboproteins. *Escherichia coli* studies suggested that the opening and closing of the tunnel could be an inherent part of the ribosome, which could, in part be regulated by the L4 and L22 proteins. As L4 and L22 riboproteins are an integral part of the ribosome binding site of macrolides and ketolides it is then logical to suggest that resistance to these agents may also be mediated by changes in both or either of L4 and L22 (Gabashvili *et al.*, 2001).

Mutations in the L4 riboprotein and L22, to a lesser extent, have been associated with macrolide and, on occasion, telithromycin resistance either alone or in combination with 23S rRNA mutations or the presence of an *ermB* gene. The most common mutation identified clinically to date in *S. pneumoniae*, is a $_{69}$GTG$_{71}$ to $_{69}$TPS$_{71}$ amino acids change. These mutations have lead to high-level erythromycin resistance in *S. pneumoniae* isolated in Eastern Europe (Nagai *et al.*, 2002). One strain harbouring these mutations was also isolated from Canada. The Canadian strain not only had the L4 mutation but also contained an *ermB* gene with a 10 amino acid truncated leader peptide (Tait-Kamradt *et al.*, 2001). This strain was reported to have high-level telithromycin resistance.

Other mutations in the L4 riboprotein identified in clinical isolates were an amino acid mutation at position 20 from serine to asparagine in combination with an

A2062C mutation in all 4 23S rRNA alleles (Depardieu & Courvalin, 2001). This strain was resistant only to 16-membered macrolides and not to 14- or 15-membered macrolides. Two insertions of amino acids into L4 have also been identified in clinical *S. pneumoniae*; a GTGREK insert at amino acid 72 (Tait-Kamradt *et al.*, 2000a) and an RRQ insert at amino acid 68 (Nagai *et al.*, 2002). The region of L4 from amino acid 63 to 74 is highly conserved in different species of bacteria.

In laboratory derived strains other L4 amino acid mutations were also described. The mutants had been passaged in azithromycin. One strain had a G69C mutation and another an SQ insertion at amino acid position 68 (Tait-Kamradt *et al.*, 2000b). A third laboratory-derived mutant had an L4 G71R mutation in combination with an L22 G95D change (Canu *et al.*, 2002). These mutants were described as macrolide resistant but their erythromycin MICs would not be classified as resistant.

From the studies carried out to date it appears that the L4 amino acid region from 67 to 72 is the hotspot for mutations conferring macrolide resistance. The mutations from GTG to TPS do not result in a change in the charge of the amino acids. Both the GTG and TPS sets of amino acids have no charge and therefore cannot be involved in the electrostatic binding to the RNA. However, both threonine and serine have aliphatic hydroxyl side chains whereas glycine does not and proline a secondary rather than a primary amino group. These subtle changes could be responsible for an alteration in the L4 conformation, which would prevent the macrolides binding and thus cause macrolide resistance.

Mutations in L22 associated with macrolide resistance have been mainly described in laboratory-derived strains of *S. pneumoniae*. In fact, only two types of L22 mutation have been identified in clinical isolates. These are a G95D amino acid change, which together with an A2059G mutation in all 4 23S rRNA alleles resulted in erythromycin MICs of 64mg/L to 128mg/L (Farrell *et al.*, 2002) and a six amino acid insertion (RTAHIT) at amino acid 109, this occurred during therapy and the patient later died (Musher *et al.*, 2002).

The G95D amino acid mutation has also been described in laboratory-derived macrolide resistant and sensitive *S. pneumoniae*. The resistant strains were selected on erythromycin and roxithromycin and had erythromycin MICs of 1mg/L and 0.25mg/L respectively. These strains had a G95D mutation alone. Other strains had two-fold mutations, a G95D mutation in L22 and a G71R change in L4, as previously mentioned. The second double mutations were an A93E mutation in L22 and C2611A mutations in 3 of the 4 23S rRNA genes, which resulted in erythromycin resistance. A P99Q change in L22 failed to render the cells macrolide resistant. A triple mutation of A93E, P91S and G83E also failed to produce erythromycin resistant strains. When the L22 genes conferring the G95E, A93E and the triplet mutations were each transformed into the macrolide sensitive CP1000 none of the resulting transformants were macrolide resistant. Although some of the L22 mutations did not confer erythromycin resistance they did cause an increase in erythromycin MIC of either 8-fold or 16-fold (Canu *et al.*, 2002).

Three strains selected on telithromycin were identified recently with 2 types of L22 mutations; one strain had a G95D mutation and the others an A97D change. These strains were not telithromycin resistant but the mutation did cause the telithromycin MIC to increase 32-fold for the G95D mutant and 8- or 16-fold for the A97D mutants (Sutcliffe *et al.*, 2000).

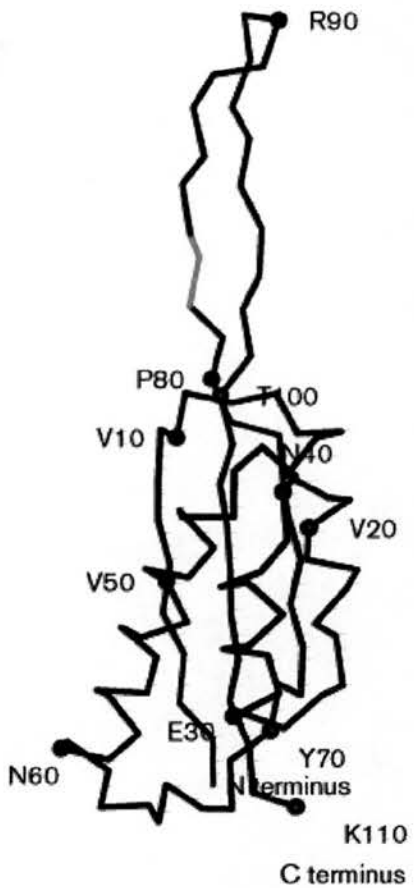


Figure 8. An α-carbon trace of the L22 riboprotein of *Thermus thermophilus* from Unge *et al.*, 1998).

The L22 protein crystal structure of *Thermus thermophilus* has been deduced and published by Unge *et al* (1998) as shown in figure 8. The tip of the L22 is mainly

positively charged. This most likely interacts with the negatively charged RNA. The amino acid residues in *T. thermophilus* contributing to this positive charge are three arginines at positions 88, 90 and 92 (Unge *et al.*, 1998). By sequence comparison in *S. pneumoniae* the amino acids at corresponding positions are an arginine at amino acid 88, a lysine at 90 and a serine at 92. Although serine is not charged, arginine and lysine are both positively-charged and would, therefore, also interact with the RNA. The area between amino acids 83 and 99 has been so far the main region of mutation. It is interesting to note that all the mutations described to date, except one, resulted in a charge change from an uncharged amino acid to a negatively-charged amino acid. How important this charge change is, remains to be seen. The amino acid mutations also conferred conformational changes on the structure of the L22 protein, which could in turn perturb the assembly of the 50S ribosomal subunit due to mutations affecting the RNA-protein recognition.

There have only been a small number of reports regarding alterations in the L22 riboprotein responsible for macrolide resistance in *S. pneumoniae*. However, the importance of these mutations is not only to determine resistance mechanisms but also to elucidate the ways in which antimicrobial agents interact with bacteria in order to devise new and more effective antimicrobial agents to overcome these resistant bacteria.

With respect to the identification of ribosomal mutations and association of specific nucleotide changes or amino acid alterations in the ribosomal proteins, the findings in *S. pneumoniae* are at a very early stage. The first mutation in the 23S rRNA genes

associated with increases in macrolide MIC and the L4 protein were published in August of 2000 (Tait-Kamradt *et al.*, 2000b) and mutations in L22 were first published in September of 2000 (Sutcliffe *et al.*, 2000). Therefore, although there have only been few strains isolated with ribosomal mutations responsible for macrolide resistance this has only been recently investigated over the past two years. The topic of macrolide resistance in *S. pneumoniae* has recently been reviewed (Leclercq & Courvalin, 2002).

1.5 Factors contributing to resistance development

The problem of antibiotic resistance overcomes the boundaries of countries and has managed to infiltrate every environment in which people live. This widespread dissemination is due not only to the practices and advances of medicine but has been influenced also by social, economic and industrial changes brought about over the past century. The bacteria involved have managed to adapt and evolve to overcome the antibiotics but we too have helped in their adaptation. Although the problem of antibiotic resistance has been realised since their development and use, the origin and evolution of antibiotic resistance and its determinants has not received much attention.

1.5.1 Origin and development

The development of antibiotic resistance is one of the best current examples of evolution. The most popular theory on the development of antibiotic resistance to date has been that antibiotic resistant bacteria arose by their selection following the use of large amounts of antibiotic. This is one theory but others do also exist, which also explain where resistant bacteria have come from.

i. Selective compartments

Generally, it has been assumed that resistant bacteria emerge due to high-level concentrations of antibiotics, which inhibit sensitive bacteria and allow resistant bacteria, not inhibited by the antimicrobial agent, to emerge and develop. Another theory suggests that selective compartments of varying concentrations of antimicrobial agent may select resistant bacteria over time (Bacquero & Blazquez, 1997; Bacquero & Negri, 1997). In classical Darwinism, the accumulation of successive minor changes by natural selection may lead to improved fitness. The transition of housekeeping genes in bacteria to high-level resistance genes probably follows this Darwinism logic. This would require successive generations of selection. Antimicrobial agents used in the human body reach a high diversity of concentration gradients in different compartments of the body. These gradients are created by the pharmacokinetics of the drugs. The variant bacteria can be selected in each compartment only within a narrow range of drug concentrations, but the range may vary depending on the populations present. If the bacterial population is serially selected then the number of variations increase such that the bacteria survive even high-level resistance. These genetic advantages may then also be transferred to other bacteria if they are incorporated onto mobile elements.

ii. Selective pressures

The main source of selective pressure for antibiotic resistance is generally thought to be antimicrobial agents. This is certainly true for antibiotic producing bacteria but antibiotics are not necessarily required to select for antibiotic resistant bacteria *in vivo* or in the environment.

A variety of compounds may be effluxed out of the bacterial cell. Such compounds including heavy metals, biocides, organic solvents and detergents are capable of selecting bacteria with increased expression of multi-drug resistance determinants. Mobile elements may also contain a variety of selective markers including antibiotic resistance genes (Alonso *et al.*, 2001). Heavy metal resistance genes and antibiotic resistance genes are often encountered together in environmental bacteria. A macrolide resistance determinant, phosphotransferase *mphBM*, is flanked by cadmium resistance efflux pump gene *cadA* in *Stenotrophomonas maltophilia* (Alonso *et al.*, 2000). Selection of these resistant bacteria may have occurred as a result of heavy metal antibiotic selective pressure in the environment, prior to human infection by these bacteria.

Furthermore, human activities also cause dramatic changes in the environmental bacterial populations. Modern farming practices require the use of probiotics and antibiotics. Links have been made between the use of avoparcin antibiotic as a growth promoter and vancomycin-resistant enterococci in the human population (Van Den Boggard & Stobberingh, 2000). The ban of avoparcin in animal feeding

has curbed the development of resistance in European Union countries (Bager *et al.*, 2000). However, antibiotic use alone is not the only link to human resistance development as the type of antibiotic used is also important. If the antibiotic gets broken down into harmless compounds on contact with the environment then it does not have the opportunity to select resistant bacteria. However, environmentally stable drugs such as the quinolones do pose a threat to humans and their bacteria. Environmental bacteria may be readily exposed to these antimicrobial agents and so select for resistant bacteria (Grave *et al.*, 1996).

Finally, multidrug efflux systems may also be regulated by stress conditions such as osmotic or oxidative shock, the presence of bile salts or medium-chain fatty acids or iron starvation as with the MexAB-Opr-D multidrug efflux system of *Pseudomonas* species (Ma *et al.*, 1995). High-level antibiotic resistance may develop in these bacteria when introduced into an environment of low iron, or other factor, as the population evolves to optimise its pumps for multipurpose functions.

iii. Medical pressure

The most prevalent theory on the emergence or reason for antibiotic resistance is that resistance in pathogenic bacteria has developed due to the use and abuse of antimicrobial agents. Macrolide consumption and macrolide resistance vary for different countries, as does the variety of macrolides available. Using the data produced from the Alexander Project (1992-1996), it can be seen that countries with the highest consumption of macrolides have the highest percentage of macrolide

resistance in *S. pneumoniae* (Bacquero, 1999). Conversely, in Finland a reduction in macrolide consumption from 2.40 to 1.38 defined daily doses per 1000 population in 1 year was followed by a decrease in resistance in *S. pyogenes* from 17% in 1992 to 9% in 1996 (Seppälä *et al.*, 1997). The link between macrolide consumption and resistance does depend on the type of macrolide prescribed. Macrolides with shorter half-lives appeared to have no correlation with the increased resistance levels, whereas a high correlation between resistance and long-acting macrolides e.g. clarithromycin prescribing, was noted by Bacquero (1999). Despite this information there has been a tendency to switch from erythromycin to clarithromycin prescribing in recent years. Therefore, careful consideration of the individual drug, as well as the class of drug must be taken to prevent or alleviate the numbers of resistant pathogens.

1.5.2 Emergence of Resistance

The emergence of resistance in bacteria as a whole can be examined from two points of view, that of the human resistance influences and the bacterial changes that have occurred to overcome antibiotics.

i. Human

The main factor of human influence is the use and misuse of antimicrobial agents which has already been discussed in relation to the prescribing practices and resistance. In this section the focus will be on the social and economic influences affecting antibiotic resistance. In 1992 the Institute of Medicine USA report (Institute of Medicine, 1992) on factors influencing emerging infectious disease identified 5 main factors, which contributed to the emergence of infectious disease these include changes in human demographics, changes in technology and industry, economic development and land use, international travel and commerce and a breakdown of public health. These influences are also contributing, to varying effects, to the emergence of antimicrobial resistance either by affecting antimicrobial use and/or the transmission of infectious diseases.

a. Changes in human demographics

Changes in demographics include an increasing percentage of people susceptible to disease requiring antibiotic treatment in the population in the developed world, an increase in the use of day care facilities and changing patterns of immigration. The increasing numbers of susceptible people include those over the age of 65, those with compromised immune systems due to HIV infection (Piot *et al.*, 2001) or diabetes and those who are receiving immunosuppressive drugs e.g. cancer chemotherapy (American Cancer Society, 2002).

Another demographic change has been the increased use of day care centres. This has increased due to the increase in one-parent families or families in which both parents work outside the home. The day care centre provides ample opportunity for transmission of bacteria e.g. *S. pneumoniae*, and resistant bacteria to and from young, susceptible children treated with multiple and/or broad-spectrum antimicrobial agents, which cause increased risk of carriage and infection with antibiotic resistant *S. pneumoniae* (Bogaert *et al.*, 2001).

Immigration patterns have in the past influenced the emergence of resistance in the USA. Between 1986 and 1995 a large increase occurred in immigration from countries with high-level antibiotic resistance problems e.g. over 350,000 people immigrated from Southeast Asia and India bringing with them not only tuberculosis (TB), but also multiple drug resistant TB (Centers for Disease Control and Prevention, 1996). The influence of the increase in numbers of people arriving into Western Europe from areas such as Eastern Europe with high-level antibiotic resistant *S. pneumoniae* has not yet been quantified but this could potentially increase the levels of antibiotic resistant *S. pneumoniae* in Western European countries.

b. Changes in technology

There has been increased development of medical technology and products over recent years. The medical technology and products currently available have helped the emergence of antibiotic resistant microbes. These advances have prolonged the lives of people and their hospitalisation after diseases, organ failure or severe

injuries, which require antibiotic treatment, thus increasing antibiotic use and infection with antibiotic resistant microorganisms. Such medical advances include the success of cancer treatment, organ transplant and survival of low-birth weight babies.

Intensive food production in the latter half of the last century and this century, relies heavily on growth promoters and antimicrobial use. Similar to day care centres, many animals, especially young animals, are crowded together. This results in the transmission of bacteria requiring antibiotic treatment and thus antibiotic resistant bacteria emerging.

c. Economic development and land use

Worldwide population growth is leading to serious environmental changes and pollution. The increase in population density not only increases the transmission rate of drug resistant bacteria but also puts pressure on the systems such as water and sanitation. The emergence of these problems may not currently affect resistance but they could lead to an increase in the variety of bacterial infections, both in the number of infections and the species of bacteria associated with the infections, which would in turn require yet more antimicrobial agent use.

d. International travel and commerce

The ease with which people and products travel around the world also means antibiotic resistant bacteria may too travel with such ease. An example of this is the clonal spread of antibiotic resistant bacteria such as penicillin resistant, serotype 23F *S. pneumoniae*, travelling from Spain to the rest of Europe and the world.

e. Breakdown of public fear

Since the introduction and use of antibiotics people have become complacent about infectious disease. Changes in morbidity and mortality and faith in drugs has lead to over-confidence towards control of infectious diseases. Unfortunately, the reign of antibiotics has started to decline due to resistance. There have been few drugs developed recently with new mechanisms of action. On the most part the available "new" antimicrobial agents are derivatives of older antimicrobial agents to which resistance has already developed. The public must be made aware of the dangers of overuse, misuse and complacency towards antimicrobial agents, so that we can preserve their use until new methods of combating bacteria and antibiotic resistant bacteria can be developed.

ii. Bacteria

In recent years the molecular mechanisms used by bacteria to become resistant to antibiotics have begun to be understood. The rapid spread of resistance mediated by plasmids, phages, transposons and other mobile genetic elements have been the main focus of attention, but resistance can also develop through changes in the chromosome. As each antimicrobial agent was introduced into clinical use a gene expressing resistance to it ultimately emerged. Emergence in this context means that the resistance gene, wherever its origin, had spread enough to get itself into a strain of a species that was isolated and noticed as resistant by a clinical laboratory (O' Brien, 2002).

If a resistance gene has developed on the chromosome of a strain, then the spread of the gene and resistance depends mainly on that strain and thus is restricted by the bacterial fitness and environment of the strain. The mobile elements, however, can move the resistance gene to another strain or species (Salyers & AmabileCuevas, 1997; Reichmann *et al.*, 1997). This gene may not have been accessible previously by this new host. Integrons and transposons may move the gene into a new plasmid and then be transferred to yet more bacteria. This process extends the range of resistance and enables resistance to penetrate more environments and bacteria. Ultimately, antibiotic pressure may select not only antibiotic resistance genes, but also the mechanisms required to transfer them to other bacteria.

A resistant strain in one host selected due to antibiotic therapy is more likely to be among the strains transferred to a second host. Similar selection in the next host would increase its chances of survival and transfer again. This theory suggests that resistant strains travel from host to host that are treated with antimicrobial agents and so selected out. This trend would continue until the strain meets another resistant strain with which it competes or a different selective environment. Then, the strain with additional mechanisms of resistance would be selected when the host was treated with another drug. As the strain went through various hosts it added to its armoury of resistance elements to ensure the fittest possible. Thus, if the strain had many plasmids conferring different advantages, such as antibiotic resistance and plasmid stability, on the strain it would be continually selected. Therefore, the resistance vector emerging from the hosts would be more competitive and persistent than either the original vector or the vectors it passed along the way. The most resistant and competitive strain would emerge.

Clonal spread of resistance works on the same theory that if a chromosomal gene conferred an advantage, e.g. resistance, to the strain then it would be selected out from the susceptible population. This gene would then be transferred vertically down the generations as the cells divide, which would in turn give these strains an advantage over susceptible strains. The result would be only the resistant strains surviving and propagating. The swiftness with which bacterial antibiotic resistance has arisen and spread reflects the diversity of the bacterial gene pools, the mobility of genes across species and genus boundaries and the short generation times and large population sizes of bacteria (O' Brien, 2002).

1.5.3 Prevention of further spread and management of resistance

The greatest fear about antibiotic resistance is that it will herald the end of the antibiotic era and our current limit on infectious disease in the developed world. Solutions are needed to overcome these resistant strains. The resistance problem is not limited by country but is a worldwide threat. Therefore, the solutions must be global rather than local.

1.6 Aims

- Evaluate the *in vitro* activity of telithromycin against common respiratory tract pathogens in comparison to a wide range of antimicrobial agents.

- Develop telithromycin resistant *Streptococcus pneumoniae* mutants *in vitro* from parents with either an *ermB* or a *mefE* gene and a macrolide sensitive strain.

- Assess the mechanisms of macrolide resistance in *S. pneumoniae* for their potential to develop telithromycin resistance by:

    1. Investigating the influence of the *ermB* and *mefE* genes and their control regions on the development of telithromycin resistance in *S. pneumoniae*.
    2. Screening the 23S rRNA macrolide and ketolide binding regions of the ribosome for mutations that could explain telithromycin resistance.
    3. Exploring the L4 and L22 riboproteins for alterations associated with telithromycin binding and resistance.

Chapter 2

Material and Reagents

2.1 Bacterial strains

The bacterial strains used in this study are shown in table 1. The clinical isolates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* were collected from various centres throughout the United Kingdom, except for the macrolide resistant *S. pneumoniae* strains, which were donated by researchers in Belguim, Italy, Canada and USA. The *Chlamydophila pneumoniae* strains TW 183, ATCC 2023 and AR 39 were obtained from the American Type Culture Collection, USA and strain D 1 was donated from Ninewells hospital, Dundee.

Table 1. Isolate origin and culture collection.

Collection centre	Culture collection	Bacteria
		S. pneumoniae
Edinburgh, Royal Infirmary	Fiona Walsh, University of Edinburgh	50 (R)
Edinburgh, Royal Infirmary	Dr. T. Dorai-Schneiders, University of Edinburgh	17 (BPE)
Edinburgh, Royal Infirmary	Penicillin resistant strains from Dr. A. Bamarouf, University of Edinburgh	7 (A)
Leeds, General Infirmary	Dr. T. Dorai-Schneiders, University of Edinburgh	26 (L)
Macrolide resistant	Fiona Walsh, University of Edinburgh	29
		M. catarrhalis
Edinburgh, Royal Infirmary	Fiona Walsh, University of Edinburgh	21 (R)
Edinburgh, Royal Infirmary	Dr. T. Dorai-Schneiders, University of Edinburgh	25 (BME)
Leeds, General Infirmary	Dr. T. Dorai-Schneiders, University of Edinburgh	30 (LM)
Wales, University Hospital Cardiff	Dr. T. Dorai-Schneiders, University of Edinburgh	15 (BMW)
Bristol, Southmead Hospital	Dr. T. Dorai-Schneiders, University of Edinburgh	9 (BMB)
		H. influenzae
Edinburgh, Royal Infirmary	Fiona Walsh, University of Edinburgh	42 (R)
Glasgow, Southern General	Dr. T. Dorai-Schneiders, University of Edinburgh	58 (BHG)

The letters in brackets refer to the different culture collections.

2.2 Storage of cultures

The *S. pneumoniae* strains were stored in Todd Hewitt broth (Oxoid, UK) with 10% glycerol and sterile distilled water mix (50% v/v) at -70°C. The *M. catarrhalis* and *H. influenzae* were stored in Brain Heart Infusion broth (Oxoid, UK) also with 10% glycerol and sterile distilled water mix (50% v/v) at -70°C.

2.3 Growth media

The growth media for *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* were obtained from Oxoid and prepared according to the manufacturer's instructions. The growth media of *Chlamydophila pneumoniae* was purchased from Sigma-Aldrich, UK. The Hep-2 cells and HL cells were kindly provided by the City hospital, Edinburgh.

2.3.1 Blood and Chocolate Agar

Streptococcus pneumoniae and *M. catarrhalis* were subcultured on blood agar plates. These consisted of 95% Columbia agar base (Oxoid, UK) and 5% defibrinated horse blood (E & O laboratories, Scotland). *Haemophilus influenzae* strains were subcultured on chocolate blood agar, which also consisted of 95% Columbia agar base and 5% defibrinated horse blood but was heated at 55°C until the agar turned from blood red to chocolate brown in colour.

2.4 Chemical reagents

All chemical reagents were obtained from Sigma-Aldrich, UK unless otherwise specified.

Chapter 3

Antimicrobial Agent Susceptibility Testing

3.1 Introduction

In order to treat a bacterial infection with the appropriate antimicrobial agent we first need to know the efficacy of the drugs available and also the ability of new drugs to inhibit bacterial growth. The most common method of testing the antimicrobial effect of these drugs *in vitro* is the minimum inhibitory concentration (MIC) test. This information may then be used to decide the course of treatment or to determine if resistance to the drug is emerging. Several methods are used to test the MIC such as Epsilon test, disk diffusion test, microtitre plates and agar doubling dilution test. The two major sets of guidelines for each method are the NCCLS and the BSAC guidelines (Phillips *et al.*, 1991). In this study the BSAC guidelines and the agar doubling dilution method were used. The agar doubling dilution method consists of agar plates containing doubling dilutions of antimicrobial agent, which are inoculated with a fixed concentration of bacteria.

The environmental factors involved in MIC testing such as medium or incubation conditions can affect the *in vitro* results (Biedenbach *et al.*, 1999; Johnson *et al.*, 1999). Susceptibility testing of lower respiratory tract pathogens is often carried out in a CO_2-rich environment. The MIC of macrolides is affected by the pH of the medium (Goldstein *et al.*, 1986; Spangler *et al.*, 1994). Thus, when the MIC plates

are incubated in CO_2 the pH falls and so the macrolide MICs are elevated giving a false reading, perhaps false resistance results (Fernandes *et al.*, 1986). As telithromycin is a macrolide derivative the effect of incubation in 5% CO_2 on telithromycin MIC was investigated.

The purpose of this study was to evaluate the *in vitro* activity of telithromycin in comparison to a variety of antimicrobial agents against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*.

3.2 Materials and Methods

3.2.1 Bacterial strains

One hundred strains each of *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* were investigated. The *Streptococcus pneumoniae* were isolated from Leeds and Edinburgh, the *Moraxella catarrhalis* from Edinburgh, Leeds and Wales and the *Haemophilus influenzae* from Edinburgh and Glasgow. The control strains consisted of *Streptococcus pneumoniae* NCTC 13593, *Staphylococcus aureus* NCTC 6571, *Haemophilus influenzae* NCTC 11931 and a laboratory reference strain of *Moraxella catarrhalis*.

3.2.2 Antimicrobial Agents

Table 2. Antimicrobial agents

Antimicrobial agent	Source	% Potency	Diluent
Telithromycin	Aventis Pharma Ltd	100	Ethanol + Sterile Distilled Water (50:50)
Erythromycin	David Bull Laboratories	100	Sterile Distilled Water (SDW)
Clarithromycin	Abbott Laboratories	100	SDW
Azithromycin	Pfizer Ltd	94.4	Ethanol + SDW (50:50)
Clindamycin	Sigma-Aldrich	92	SDW
Moxifloxacin	Bayer AG	100	1M NaOH + SDW
Levofloxacin	Aventis Pharma Ltd	100	SDW
Ciprofloxacin	Bayer AG	100	SDW
Gemifloxacin	SmithKline Beecham	75.4	SDW
Linezolid	Pharmacia and Upjohn	100	Ethanol + SDW
Amoxycillin	CP Pharmaceuticals	100	SDW
Clavulanic Acid	SmithKline Beecham	94.7	SDW
Faropenem	Bayer AG	81	SDW

The antimicrobial agents were stored and prepared according to the manufacturer's guidelines. Amoxiclav consisted of fixed concentrations of 2mg/L of clavulanic acid per plate and doubling dilutions of amoxycillin.

3.2.3 Minimum Inhibitory Concentrations

The minimum inhibitory concentration was defined as the lowest concentration of antimicrobial agent required to inhibit visible growth of bacteria *in vitro*. The MICs were performed on Columbia agar base supplemented with 5% defibrinated horse blood for *Streptococcus pneumoniae* and *Moraxella catarrhalis* and on chocolate Columbia agar plates for *Haemophilus influenzae* according to the British Society for Antimicrobial Chemotherapy guidelines for susceptibility testing (Phillips *et al.*, 1991). The MICs were determined by the standard agar doubling dilution method. The MIC plates containing telithromycin were incubated both in air and 5% CO_2, both at 37°C. All other plates were incubated in air at 37°C.

The *S. pneumoniae* strains were grown overnight in 5% CO_2 on blood agar plates. They were then inoculated into 0.85% sterile saline solutions. The turbidity of *S. pneumoniae* was adjusted to 0.5 McFarland standard, which is equivalent to 10^7 cfu/mL. This was then reduced to a final concentration of 10^5 cfu per spot on the MIC plates. The *Moraxella catarrhalis* and *Haemophilus influenzae* were inoculated into Brain Heart Infusion broth and incubated in 5% CO_2 overnight. The cultures of both were diluted in 0.85% sterile saline and inoculated at a final concentration of 10^4 cfu per spot on the antibiotic plates. The plates were inoculated using a

multipoint inoculator (Denley Tech, Billinghurst, UK). A control plate containing no antimicrobial agent was included for each set of MIC tests. The inoculated petri plates were incubated for 16 to 18 hours at 37°C in air or 5% CO_2. The MIC tests were repeated at least once for each strain.

The antimicrobial agent breakpoints were according to the BSAC guidelines (MacGowan & Wise, 2001) except for telithromycin for which the NCCLS breakpoint (NCCLS, 2000) was used. Resistance to the antimicrobial agents was assigned at the following MIC values: telithromycin ≥ 4mg/L, erythromycin ≥ 1mg/L, clarithromycin ≥ 1mg/L, azithromycin ≥ 2mg/L and clindamycin ≥ 1mg/L.

3.3 Results

3.3.1 Effect of CO_2 Incubation

When the MIC plates containing telithromycin were incubated in 5% CO_2 there was an increase in MIC regardless of whether the bacteria was *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* as shown in tables 3, 4 and 5. The range and MIC_{90} for *S. pneumoniae* increased by 1 doubling dilution and the MIC_{50} increased by 2 doubling dilutions. The telithromycin breakpoint for *S. pneumoniae* is 1mg/L. The *S. pneumoniae* range endpoint increased from 1mg/L to 2mg/L when incubated in CO_2, table 3. With a telithromycin MIC of 2mg/L a *S. pneumoniae* strain is no longer considered sensitive to telithromycin. Therefore, some strains tested changed from telithromycin sensitive to non-sensitive when incubated in CO_2.

With regard to *M. catarrhalis* and *H. influenzae* the MIC_{50} and MIC_{90} both increased by 1 doubling dilution when incubated in CO_2 indicated in tables 4 and 5. The proposed telithromycin breakpoint for *H. influenzae* was 2mg/L. With the CO_2 effect the number of *H. influenzae* strains that were not sensitive to telithromycin increased from 18% to 63% so that the MIC_{90} value effectively changed to the MIC_{50} value. The incubation conditions rather than resistance mechanisms caused this decreased susceptibility to telithromycin.

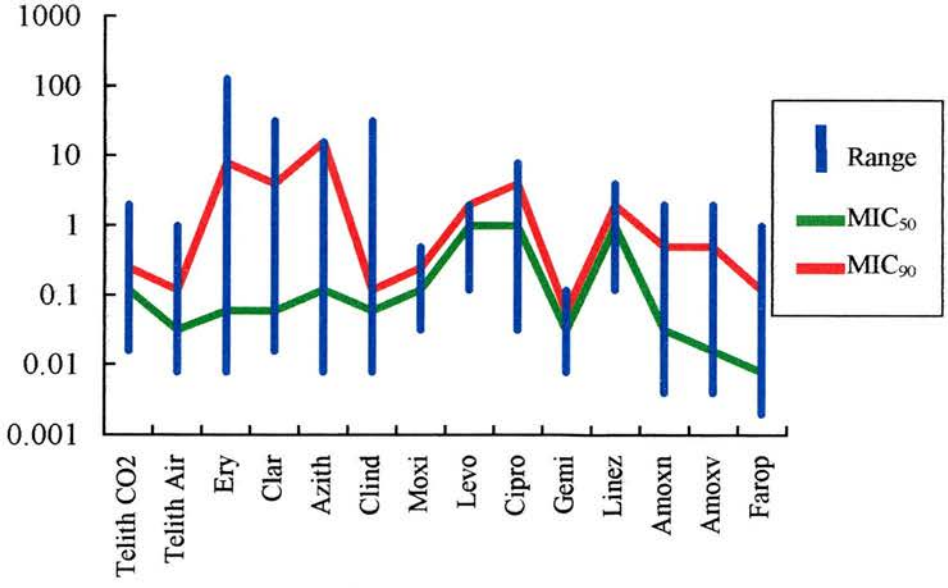
3.3.2 *Streptococcus pneumoniae*

The MIC results of *S. pneumoniae* are shown in table 3 and figure 9. Telithromycin had the second lowest MIC_{90} of all the antimicrobial agents tested against *S. pneumoniae*. It was merely 1 doubling dilution higher than that of the lowest MIC_{90}, which belonged to gemifloxacin. The closest comparators to telithromycin, by mode of action, are the macrolides. The telithromycin MIC_{90} was 5 doubling dilutions lower than the lowest macrolide MIC_{90}. The lincosamides were represented by clindamycin, which are also protein synthesis inhibitors. While the MIC_{90} of clindamycin was the same as telithromycin the upper limit of the range was substantially higher for clindamycin at 32mg/L in comparison to 1mg/L for telithromycin. The fluoroquinolones had four representatives: levofloxacin, ciprofloxacin, moxifloxacin and gemifloxacin. Gemifloxacin and moxifloxacin both had excellent activity against *S. pneumoniae* with their highest MICs at 0.12mg/L and 0.5mg/L respectively. Levofloxacin and ciprofloxacin did not perform as well and had low activity relative to telithromycin and the newer fluoroquinolones. Linezolid belongs to a new family of antimicrobial agents called the oxazolidinones. However, it did not perform as well as the other antimicrobial agents. Amoxycillin and amoxiclav both had MIC_{90}s lower than those of the macrolides but they were still higher than that of telithromycin by two doubling dilutions. Faropenem, a novel carbapenem, also had good activity against *S. pneumoniae*.

Table 3. *Streptococcus pneumoniae* MIC results

Antimicrobial Agents	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Telithromycin CO_2	0.016-2	0.12	0.25
Telithromycin air	0.008-1	0.032	0.12
Erythromycin	0.008-128	0.06	8
Clarithromycin	0.016-32	0.06	4
Azithromycin	0.008-16	0.12	16
Clindamycin	0.008-32	0.06	0.12
Moxifloxacin	0.032-0.5	0.12	0.25
Levofloxacin	0.12-2	1	2
Ciprofloxacin	0.032-8	1	4
Gemifloxacin	0.008-0.12	0.032	0.06
Linezolid	0.12-4	1	2
Amoxycillin	0.004-2	0.032	0.5
Amoxiclav	0.004-2	0.016	0.5
Faropenem	0.002-1	0.008	0.12

Figure 9. *Streptococcus pneumoniae* MICs. Minimum inhibitory concentration (mg/L) vs. antimicrobial agent.



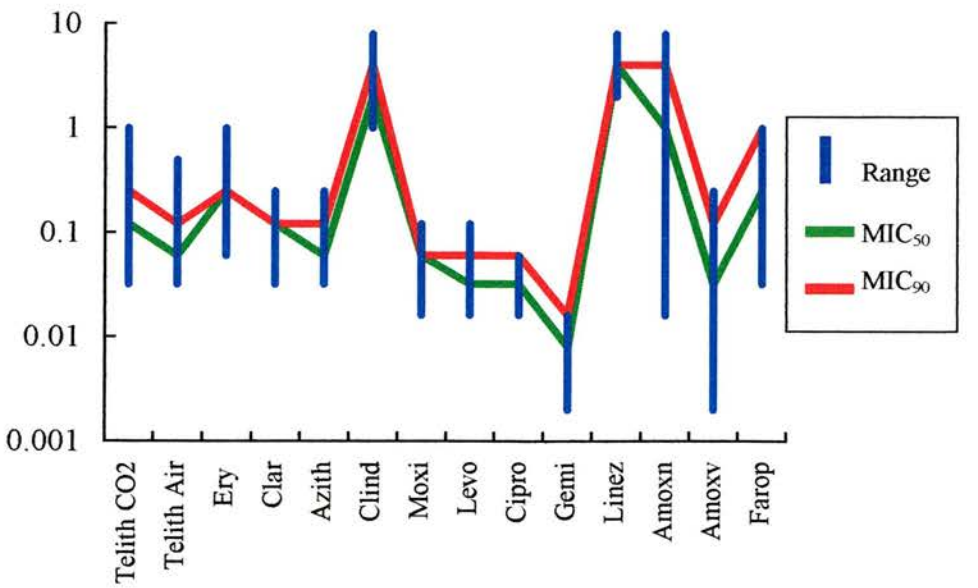
3.3.3 *Moraxella catarrhalis*

The MIC results of *M. catarrhalis* are shown in table 4 and figure 10. The fluoroquinolones had the greatest activity of the antimicrobial agents tested against *M. catarrhalis*. Their MIC_{90}s ranged from 0.016mg/L to 0.06mg/L. In comparison to this the MIC_{90}s of the macrolides were 0.12mg/L and 0.25mg/L. There was little difference between the performance of telithromyin and the macrolides. Clindamycin, linezolid and amoxycillin all had low activity against *M. catarrhalis in vitro* with MIC_{90}s of 4mg/L. However, when clavulanic acid was added to amoxycillin the potency was dramatically increased, from an MIC_{90} of 4mg/L to an MIC_{90} of 0.12mg/L. The faropenem MIC_{90} of 1mg/L was higher than that of telithromycin, the macrolides and the quinolones by at least two doubling dilutions.

Table 4. *Moraxella catarrhalis* MIC results.

Antimicrobial Agents	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Telithromycin CO_2	0.032-1	0.12	0.25
Telithromycin air	0.032-0.5	0.06	0.12
Erythromycin	0.06-1	0.25	0.25
Clarithromycin	0.032-0.25	0.12	0.12
Azithromycin	0.032-0.25	0.06	0.12
Clindamycin	1-8	2	4
Moxifloxacin	0.016-0.12	0.06	0.06
Levofloxacin	0.016-0.12	0.032	0.06
Ciprofloxacin	0.016-0.06	0.032	0.06
Gemifloxacin	0.002-0.016	0.008	0.016
Linezolid	2-8	4	4
Amoxycillin	0.016-8	1	4
Amoxiclav	0.002-0.25	0.032	0.12
Faropenem	0.032-1	0.25	1

Figure 10. *Moraxella catarrhalis* MICs. Minimum inhibitory concentration (mg/L) vs. antimicrobial agent.



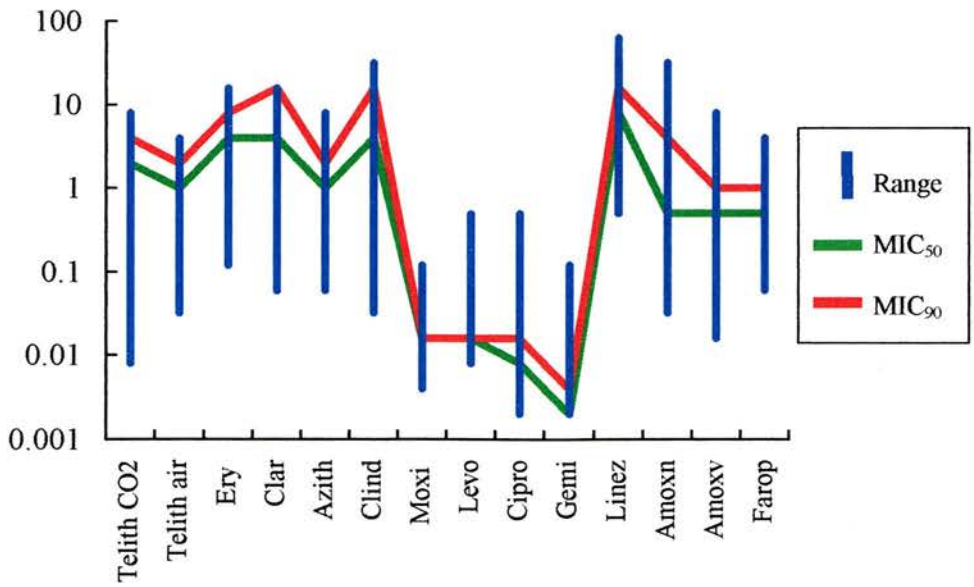
3.3.4 *Haemophilus influenzae*

The MIC results of *H. influenzae* are shown in table 5 and figure 11. Telithromycin had relatively low activity against *H. influenzae* with an MIC_{90} of 2mg/L. It had lower MIC_{50} and MIC_{90} values than erythromycin and clarithromycin by at least two dilutions. For *H. influenzae* clindamycin and linezolid had the same MIC_{90}s as clarithromycin of 16mg/L. Once again the fluoroquinolones performed with the highest activity and low MIC_{90}s of 0.004mg/L to 0.016mg/L. Amoxycillin had improved activity when combined with clavulanic acid to form amoxiclav. The MIC_{90} decreased from 4mg/L to 1mg/L. Faropenem had the same activity *in vitro* as amoxiclav against *H. influenzae*.

Table 5. *Haemophilus influenzae* MIC results.

Antimicrobial Agents	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Telithromycin CO_2	0.008-8	2	4
Telithromycin air	0.032-4	1	2
Erythromycin	0.12-16	4	8
Clarithromycin	0.06-16	4	16
Azithromycin	0.06-8	1	2
Clindamycin	0.032-32	4	16
Moxifloxacin	0.004-0.12	0.016	0.016
Levofloxacin	0.008-0.5	0.016	0.016
Ciprofloxacin	0.002-0.5	0.008	0.016
Gemifloxacin	0.002-0.12	0.002	0.004
Linezolid	0.5-64	8	16
Amoxycillin	0.032-32	0.5	4
Amoxiclav	0.016-8	0.5	1
Faropenem	0.06-4	0.5	1

Figure 11. *Haemophilus influenzae* MICs. Minimum inhibitory concentration (mg/L) vs. antimicrobial agent.



The MICs of the individual isolates of *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* are shown in appendix 1.

3.3.5 Resistance levels

Table 6. Resistance levels of telithromycin, macrolides and clindamycin.

Antimicrobial agent	*Streptococcus pneumoniae* (%)	*Moraxella catarrhalis* (%)	*Haemophilus influenzae* (%)
Telithromycin	0	0	0
Erythromycin	20	1	8
Clarithromycin	19	0	0
Azithromycin	14	0	1
Clindamycin	4	100	97

Telithromycin had the lowest resistance levels of the macrolides and lincosamide, with no resistance in *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* as shown in table 6. In comparison, about one fifth of the *S. pneumoniae* population were erythromycin and clarithromycin resistant. With regard to *S. pneumoniae* 4% were resistant to clindamycin.

In *M. catarrhalis* the entire population were found to be clindamycin resistant while one strain was also erythromycin resistant. No resistance to telithromycin, clarithromycin or azithromycin occurred.

Clindamycin resistance was also a major problem in *H. influenzae* with 97% of the strains resistant. Erythromycin resistance was at 8% for *H. influenzae*, whereas azithromycin and clarithromycin resistance levels were 1% and 0% respectively.

3.4 Discussion

Respiratory tract infections are a major cause of morbidity and mortality in the community and hospitals. *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* are common causes of lower respiratory tract infections and upper respiratory tract infections. Worldwide, the annual incidence of community acquired pneumoniae (CAP) is estimated to be 1.1-4.0 per 1000 population. In the UK CAP accounts for 5-12% of cases of LRTIs among adults approximately 20-42% of which require hospitalisation (Finch, 2001). Macrolide resistance is currently an increasing problem in *Streptococcus pneumoniae* throughout the world. France and Hong Kong have extremely high levels of resistance in community acquired *S. pneumoniae*. (Felmingham & Grüneberg, 2000)

The need for standard, reproducible methods of testing the efficacy of antimicrobial agents is widely accepted as being important. This includes the incubation conditions of the MIC plates after inoculation. This study has shown that the MIC of telithromycin increased when incubated in 5% CO_2 in comparison to air incubation. This increase could have a dramatic effect on the apparent emergence of resistance to telithromycin. From this study alone *S. pneumoniae* and *H. influenzae* strains changed from telithromycin sensitive to non-sensitive merely due to the incubation

conditions. Standardisation of conditions for MIC testing and incubation conditions are required in order to assess the true percentage of bacteria that are resistant to antimicrobial agents. There is also a need to take the effect of CO_2 into account when defining breakpoints of antimicrobial agents to ensure that the breakpoint value allows for the increase in MIC when incubated in CO_2.

In this and previous studies macrolide resistance has been associated mainly with *S. pneumoniae* rather than *M. catarrhalis* or *H. influenzae* (Felmingham & Grüneberg, 2000). In the Alexander project survey erythromycin resistance in *S. pneumoniae*, in the UK was at 13.6% in 1996 and 7.2% in 1997. The results in this chapter suggests that erythromycin resistance has increased to 20% for the *S. pneumoniae* tested from two British cities. The percentage of *H. influenzae* resistant to erythromycin is also worrying at 11%; however, almost all are clarithromycin and azithromycin sensitive.

Telithromycin has previously been shown to have activity against macrolide resistant bacteria. This finding has been borne out in the results. Telithromycin had consistently lower MICs than erythromycin against all the bacteria tested. No resistance to telithromycin for either *S. pneumoniae, M. catarrhalis* or *H. influenzae* was found. The high overall activity of telithromycin against the three respiratory pathogens and no resistance indicates that telithromycin has good potential as a respiratory tract pathogen inhibitor, even if they are already macrolide resistant.

Chapter 4

Culturing *Chlamydophila pneumoniae*

4.1 Introduction

Chlamydophila pneumoniae (formerly *Chlamydia pneumoniae*) is an intracellular pathogen responsible for respiratory tract infection. A recent British survey of the microbial aetiology of community-acquired pneumonia (CAP) indicated that *C. pneumonia* is responsible for 13% of CAP and is the second highest bacterial cause of CAP (Lim *et al.*, 2001). It has been associated with bronchitis, pharyngitis, sinusitis, myocarditis, endocarditis and coronary artery disease (Grayston *et al.*, 1990).

In order to treat *C. pneumoniae* infections it is first essential to know the efficacy of the available antimicrobial agents against this pathogen. Minimum inhibitory concentration (MIC) tests are used to investigate the *in vitro* capabilities of antimicrobial agents on bacteria. Therefore, MIC testing of *C. pneumoniae* needs to be carried out before an antimicrobial agent may be used to treat the infection. Culturing of *C. pneumoniae* must occur before MIC tests can be performed. *Chlamydophila pneumoniae* is known to be very difficult to culture and is far more difficult than other chlamydial species (Grayston *et al.*, 1990). Many methods of

culturing *C. pneumoniae* have been proposed with differing cell lines, centrifugation conditions and incubation times.

There is no standard method for culturing *C. pneumoniae* nor for testing the MIC of antimicrobial agents against it. However, a meeting was recently convened by the Centers for Disease Control and Prevention (Atlanta, USA) and the Laboratory Centre for Disease Control (Ottawa, Canada) to review current diagnostic tests and procedures used with *C. pneumoniae* and provide recommendations for standardised approaches (Dowell *et al.*, 2001). The recommendations for the culture conditions that should be used however, suggested two different cell lines and centrifugation conditions varying from 900g to 3000g. There is no agreement on optimal culture conditions between different laboratories and even within the same laboratory. For *C. pneumoniae* to be a viable organism in the sense that it may be tested with regularity *in vitro*, a reliable method of culturing is required.

The initial aim of this investigation was to culture *C. pneumoniae* and test the efficacy of telithromycin in comparison to macrolides against *C. pneumoniae*. After some difficulty was had in culturing *C. pneumoniae* this aim changed to firstly culturing *C. pneumoniae* using previously published methods.

4.2 Materials and Methods

4.2.1 Bacterial strains

The three strains TW 183, ATCC 2023 and AR 39 were from the American Type Culture Collection, the fourth strain, D 1, was received from Ninewells hospital, Dundee. The D 1 strain had been successfully cultured using an in-house culture method in Dundee. All strains were stored at -70°C.

4.2.2 Cell lines

Hep-2 cells (City Hospital, Edinburgh) were cultured in Eagles Minimum Essential Medium (EMEM) supplemented with sodium bicarbonate, 5% denatured foetal bovine serum, 2mM L-Glutamine, 10% tryptose phosphate broth, 1% amphotericin B, 1% streptomycin and 1% vancomycin.

HL cells (sourced from the Washington Research Foundation) were grown in Dulbeccos Modified Eagles Medium with 2% of 1M HEPES buffer, 1.5% of sodium bicarbonate, 10% denatured foetal bovine serum, 1% L-Glutamine, 1% vancomycin and 1% gentamicin.

Vancomycin was obtained from Edinburgh Royal Infirmary hospital pharmacy and Fisher Scientific, UK, supplied cycloheximide. All the other reagents used in these experiments were obtained from Sigma-Aldrich Co., UK.

4.2.3 Harvesting and Passaging Cells

A monolayer of Hep-2 cells and a monolayer of HL cells were grown in 75cm^2 tissue culture flasks and passaged weekly using aseptic techniques. The growth medium was poured off and the cell layer washed 3-4 times with approximately 20mL of sterile phosphate buffered solution (PBS). One millilitre of versene/trypsin (0.5%/1%) was added and incubated at 37°C for one minute. The versene/trypsin mixture was poured off and the cells left for 5-10 minutes. The cell sheet was examined periodically and when the sheet started to detach from the flask wall 10mL of growth medium was pipetted into the flask. The flask wall on which the cells were growing was rinsed and the cells pipetted up and down several times with minimal frothing to break up any clumps of cells. The cells were counted using an Improved Neubauer Counting Chamber and diluted in growth medium as appropriate for further use. If the cells were to be used to seed 75cm^2 flasks then 1.5 x 10^{6}cells/ml were required but if shell vials were seeded 0.5 x 10^{6}cells/ml were added to each shell vial. The cells were regularly passaged and incubated at 37°C.

4.2.4 *Chlamydophila pneumoniae* culture in Hep-2 cells

Two different inoculation methods of Hep-2 cells with *C. pneumoniae* were attempted. The first procedure was adapted from Sriram *et al* (1999) and the second was adapted from Roblin *et al* (1992).

1. A monolayer of Hep-2 cells was grown up in shell vials for either 24 or 48 hours prior to inoculation. The *C. pneumoniae* strains were diluted from 1 in 100 to 1 in 10^9 in EMEM growth medium. 100µl of each dilution were added to 400µl of EMEM growth medium in the shell vial. These were centrifuged at 4°C and 1800g for 1 hour. The medium was decanted and replaced with 1ml of growth medium containing 20% denatured foetal bovine serum, 4µg/ml cycloheximide and 4mM L-Glutamine in each shell vial. These were incubated for 7 days at 37°C with further centrifugation on days 4, 5 and 6.

2. The same cells and dilutions were used as method 1. The shell vials were centrifuged at 37°C and 1700g for 1 hour and incubated at 37°C for 1 hour. The medium was decanted and replaced with 2mls of growth medium with 10% denatured foetal bovine serum and 1 µg/ml cycloheximide for each shell vial. They were incubated at 37°C for 72 hours.

Negative controls consisted of a shell vial containing uninoculated cells and were included in each run. All the negative controls were processed before processing those thought to contain *C. pneumoniae*.

4.2.5 *Chlamydophila pneumoniae* culture in HL cells

HL cells had previously been inoculated in Dundee with D 1 using this procedure. A monolayer of HL cells was grown up in shell vials for 24 or 48 hours prior to inoculation. The growth medium was removed from the shell vials and the cells rinsed with 1-2mL of sterile PBS. Each shell vial was inoculated with 300μL of each of the strains. The vials were then centrifuged at 2400g at 35°C for 1 hour. The medium was removed and replaced with HL medium containing 1.3 μg/mL cycloheximide but without vancomycin and gentamicin. The vials were incubated at 37°C for seven days with additional centrifugations on days 3, 4 and 5. On day seven the vials were sonicated in an ultra sonicating water bath. Three hundred microliters of the sonicated medium was added to freshly prepared monolayers and the procedure was repeated. All the negative controls were processed before processing those thought to contain *C. pneumoniae*.

Shell vial coverslips from each passage of each method were tested for the presence of *C. pneumoniae* inclusions. The cultures were fixed and stained using direct and indirect antibody tests. The direct antibody test utilised a genus-specific monoclonal antibody to *Chlamydia* lipopolysaccharide antigen present in all known strains of

Chlamydiae and a specific direct antibody test for *C. pneumoniae*. These tests were DAKO Imagen™ tests. The test was carried out according to the manufacturer's instructions. The indirect test utilised a mouse monoclonal anti-*Chlamydia pneumoniae* antibody and a rabbit anti-mouse fluorescent isothiocyanate conjugated (FITC) antibody. Both antibodies were diluted in PBS according to the manufacturer's instructions. The indirect identification procedure was repeated but rabbit serum was also added at the same time as the mouse monoclonal anti-*Chlamydia pneumoniae* antibody to block the excess rabbit anti-mouse FITC antibody. Inclusions when present should appear as apple-green circles within the cell.

4.3 Results

The four *C. pneumoniae* strains were succesively passaged in varying growth conditions, each time one parameter would be altered so as to optimise the culture technique. However, no inclusions were found in any of the shell vials tested. The cells were washed a varying number of times and the growth medium and PBS were filtered in order to eliminate bacterial contamination, these steps were carried out each time the cells, growth medium and PBS were used. Each of the three different methods were implimented using the four different strains and two different cell lines. For each coverslip recovered two different staining procedures were used but neither resulted in identification of a single *C. pneumoniae* inclusion. If the ATCC

strains had been non-viable then the strain from Dundee should at least have provided a positive result.

4.4 Discussion

The lack of inclusions suggests that either the *C. pneumoniae* were not viable or the methods used were not easy to reproduce. As the D 1 strain had previously been cultured this strain was viable and so this strain at least should have resulted in the identification of inclusions. This lack of reproducibility is a common problem with *C. pneumoniae*.

Development of new methods will not alter the problem of *C. pneumoniae* culturing. Standardisation of the existing methods so that they are reproducible not only in the same laboratory but also in different laboratories is the only way that data, particularly information about the antibiotic profile of *C. pneumoniae*, may be generated. Therefore, standard, reproducible methods, which are agreed upon by more than one laboratory are needed not only for culturing but also for the investigation of antibiotic resistance in *C. pneumoniae.* A working party of *Chlamydia* scientists is needed that will create culture guidelines and antimicrobial agent testing guidelines with defined parameters similar those created by the BSAC or NCCLS. Thus accurate and valid information on culturing would be available.

Chapter 5

Mutation Studies

5.1 Introduction

Acquisition and further spread of antibiotic resistance determinants and selection of resistant strains among virulent bacterial populations is the most pressing problem for the treatment of infectious disease. There are different theories on the emergence of antibiotic resistant bacterial strains. Theory 1: the bacteria acquire resistance genes, for instance by plasmid-mediated transfer, which allow the hosts to survive in the presence of the antimicrobial agent. Theory 2: bacteria within the population have a selective advantage, such as an efflux pump, which prevents the antimicrobial agent taking effect and so are preferentially selected out from a population that normally does not contain such mechanisms. In this case, the antibiotic does not cause resistance but rather provides an environment that selects for certain strains. Theory 3: the presence of an antimicrobial agent causes a chemical or metabolic change in the bacteria, which leads to antibiotic resistance. How and by which method bacteria overcome the effect of antibiotics is still not fully elucidated.

Regardless of the mechanism used to achieve resistance, mutation studies are efficient *in vitro* methods to select resistant strains so that further investigation of

resistance can be performed. Mutation studies are therefore the first step required to identify the changes that occur along the pathway from a sensitive strain to a non-sensitive strain and finally a resistant strain.

5.2 Material and Methods

5.2.1 Bacterial strains

Twenty-nine erythromycin-resistant *Streptococcus pneumoniae* were collected from the USA, Canada, Belgium and Italy. They were either macrolide, lincosamide and streptogramin B (MLS_B) resistant or resistant only to macrolides. The strains 02J1095, with an *ermB* gene, 02J1175, with a *mefA* gene and an MLS_B sensitive strain NCTC 13593 were used as the parents for the step-wise selection of telithromycin resistant mutants.

5.2.2 Minimum Inhibitory Concentrations

The MIC of the macrolide resistant strains were tested according to the BSAC guidelines but all plates were incubated in 5% CO_2 (Phillips *et al.*, 1991). The antimicrobial agent breakpoints were according to the BSAC guidelines (MacGowan & Wise, 2001) except for telithromycin, for which the NCCLS breakpoint (NCCLS,

2000) was used. Resistance to the antimicrobial agents was assigned at the following MIC values: telithromycin ≥ 4mg/L, erythromycin ≥ 1mg/L, clarithromycin ≥ 1mg/L, azithromycin ≥ 2mg/L and clindamycin ≥ 1mg/L.

5.2.3 Mutation Studies

Each of the three parent strains were inoculated into Todd Hewitt broth and incubated in 5% CO_2 overnight. Varying dilutions of the cultures were spread on Columbia agar plates supplemented with 5% defibrinated horse blood containing telithromycin at concentrations equal to the MIC or 2 x MIC of the strain. Each concentration was carried out in triplicate for the MIC, the 2 x MIC and the control with no antibiotic. All plates were incubated in 5% CO_2 for 48 hours. The resulting mutants were subcultured twice on plates with the appropriate telithromycin concentration of either the MIC or 2 x MIC. The MICs of telithromycin against the mutants were tested at this point according to the BSAC guidelines (Phillips *et al.*, 1991). Successive generations were derived in the same way as the first generation. This process was repeated until high-level telithromycin resistance occurred or the MICs of successive generations remained the same. The stability of a representative of each generation was investigated by serially subculturing the mutants on antibiotic-free medium for 10 generations and then retesting their telithromycin MICs.

5.3 Results

5.3.1 MICs of Macrolide Resistant Strains

The MICs of the 29 macrolide resistant clinical isolates were tested. The results are shown in table 7. As there were only 29 isolates the MIC_{50} and MIC_{90} values were not calculated. The individual strain MICs are detailed in appendix 2.

Table 7. Macrolide resistant *S. pneumoniae* MIC results.

	Telith	Ery	Clar	Azith	Clind
Range (mg/L)	0.032-2	4->64	1->64	8->64	0.06->32
Median (15)	0.12	>16	>16	>32	>16
% Resistance	0	100	100	100	69

All 29 *S. pneumoniae*, which were erythromycin resistant, were not resistant to telithromycin (table 7). Three strains were, however, not sensitive to telithromycin with MICs of 2mg/L. All three had macrolide and lincosamide (ML) resistance phenotypes as they were resistant to macrolides and the lincosamide clindamycin. These strains were all from Italy. It is most probable that the clindamycin-sensitive strains were *mefA* positive and thus were macrolide resistant by the efflux mechanism. Telithromycin showed good activity against both the macrolide (M) and ML resistance phenotypes. There does not appear to be a correlation between the M

or ML resistance phenotype and telithromycin resistance, although the three telithromycin non-sensitive strains were all of the ML phenotype.

5.3.2 Mutation studies

Telithromycin resistant mutants were created from the macrolide resistant strains 02J1095 and 02J1175. The plating of the macrolide sensitive NCTC 13593 strain on agar containing telithromycin from concentrations of 0.016mg/L for the first generation to 0.5mg/L for the fourth generation, failed to produce telithromycin resistant mutants, as shown in table 10. The NCTC 13593 strain, which was macrolide sensitive, had a telithromycin MIC of 0.016mg/L. This increased sequentially to 0.032mg/L, 0.12mg/L, 0.5mg/L and 0.5mg/L for the four mutant generations respectively. There was decreased susceptibility to telithromycin with an increase in telithromycin MIC from 0.016mg/L for the parent strain to a telithromycin MIC of 0.5mg/L for the third and fourth generation mutants. This is greater than a 20-fold increase in MIC over three generations.

The MIC of 02J1095 and its mutants increased from 0.06 mg/L for the parent to >32mg/L over two generations, as illustrated in table 8. The first generation contained one strain with a telithromycin MIC of 8mg/L. The second generation which had all been selected on agar which a telithromycin concentration of 1mg/L were highly telithromycin resistant with MICs ranging from 4mg/L to >32mg/L.

With regard to 02J1175, the telithromycin MIC of the parent strain was 0.5mg/L. This increased over four generations to 2mg/L, 4mg/L and 8mg/L respectively, as shown in table 9. While the increase in the telithromycin MIC was not as marked as for strain 02J1095 the telithromycin MICs did increase sequentially from 0.5mg/L to 8mg/L. The final generation strain M IV was selected from an agar plate with 8mg/L but also had a telithromycin MIC of 8mg/L.

Table 8. Mutation study results of 02J1095.

Parent	Gen 1	Gen 2
02J1095 (0.06)	J II 1-10 (0.06-1) J II 1-8 [0.06] J II 9, J II 10 [0.12]	J III 1-9 (4->32) J III 1-9 [1]

( ) = Telithromycin MIC (mg/L)

[ ] = Telithromycin plate concentration that the strain was selected from (mg/L).

Gen = Generation

Table 9. Mutation study results of 02J1175

Parent	Gen 1	Gen 2	Gen 3	Gen 4
02J1175 (0.5)	M I 1-10 (1-4) M I 1-10 [0.5]	M II 1-18 (2-4) M II 1-10 [1] M II 11-18 [2]	M III 1-3 (8) M III 1-3 [4]	M IV (8) M IV [8]

( ) = Telithromycin MIC (mg/L)

[ ] = Telithromycin plate concentration that the strain was selected from (mg/L).

Gen = Generation

Table 10. Mutation study results of NCTC 13593.

Parent	Gen 1	Gen 2	Gen 3	Gen 4
NCTC 13593 (0.016)	N I 1-10 (0.016-0.032) N I 1-10 [0.016]	N II 1-9 (0.032-0.12) N II 1-4 [0.032] N II 5-7 [0.06] N II 8-9 [0.032]	N III 1-12 (0.12-0.5) N III 1-6 [0.12] N III 7-12 [0.25]	N IV 1-5 (0.25-0.5) N IV 1-5 [0.5]

( ) = Telithromycin MIC (mg/L)

[ ] = Telithromycin plate concentration that the strain was selected from (mg/L).

Gen = Generation

5.3.3 Mutation Frequencies

The frequency of mutation is highest overall for the NCTC 13593 mutants, as indicated in table 11. The lowest frequency of NCTC 13593 mutants per population was 1 in 100. Although this is extremely high, no mutants could be created with a telithromycin MIC of higher than 0.5mg/L. The first generation mutation frequency of the 02J1095 mutants was 1 mutant per 1000, as shown in table 11. The second generation mutation frequency was 1 per 10^6. This is obviously less frequent than the first generation but the fact that a jump in MIC of such magnitude can occur is of interest. These increases were stable, such that when the mutants were serially subcultured 10 times without the selective pressure of telithromycin the MIC of telithromycin for each strain did not decrease. The first generation mutation

frequency of 02J1175 mutants was 2 per 10,000 (table 11). This decreased to 3 per 100,000 for the second generation and 3 per million for the third generation. Interestingly, after the third generation, mutants were selected more readily at a frequency of 3 per 100,000. However, when the final generation strains were subcultured they did not grow well and as a result only one strain survived as a fourth generation mutant.

Table 11. Mutation frequencies.

Strain	Telithromycin MIC (mg/L)	Generation	Mutation frequency
02J1095 (*ermB*)	0.06	Parent	
J II 1	1	1	1×10^{-3}
J III 1	4	2	1×10^{-6}
J III 8	>32	2	1×10^{-6}
02J1175 (*mefA*)	0.5	Parent	
M I 2	2	1	2×10^{-4}
M II 15	4	2	3×10^{-5}
M III 3	8	3	3×10^{-6}
M IV	8	4	3×10^{-5}
NCTC 13593	0.016	Parent	
N I 1	0.032	1	2×10^{-2}
N II 7	0.12	2	3×10^{-2}
N III 11	0.5	3	6×10^{-1}
N IV 3	0.5	4	2×10^{-2}

5.3.4 Revertant Strains MICs

Representative mutants were serially subcultured ten times without the selective pressure of telithromycin to investigate if the mutants required the presence of telithromycin in order to maintain the elevated telithromycin MICs or if the mechanism used by the bacteria to prevent telithromycin activity persisted even in the absence of telithromycin. There was no decrease in the telithromycin MIC of J II 1, J III 1 or J III 8, with telithromycin MICs of 1mg/L, 4mg/L and >32mg/L respectively, after serial passage. There was also no decrease in the telithromycin MIC of selected NCTC 13593 mutants with telithromycin MICs of 0.06mg/L, 0.12mg/L and 0.25mg/L. Therefore, the 02J1095 and NCTC 13593 mutants with elevated telithromycin MICs had stable mechanisms of preventing telithromycin activity, which did not require the selective pressure of telithromycin.

Table 12. Revertant telithromycin MICs.

Parent		⟶	Revertant	
Telithromycin MIC (mg/L)			Telithromycin MIC (mg/L)	
M I 2	2		M I	0.5
M II 15	4		M II	2
M III 3	8		M III	4
M IV	8		M IV	1

The 02J1175 mutants selected with telithromycin MICs from 2mg/L to 8mg/L were not stable (table 12). When the mutants were serially subcultured on telithromycin-free agar ten times, the resulting strains differed from the original strains in that they had increased susceptibility to telithromycin. The MIC of telithromycin for the most resistant strain M IV reverted from 8mg/L to 1mg/L after 10 passages in antibiotic-free medium. The MIC of each generation representative also decreased but not as dramatically as the fourth generation mutant. For the third generation strain, M III 3, the decrease in telithromycin MIC meant that it remained telithromycin resistant with an MIC of 4mg/L. When the M IV reverant was mutated on agar plates containing either 1mg/l or 2mg/l all the resulting mutants returned to an MIC of 8mg/l, the same as M IV. The M IV strain was selected from agar plates with a telithromycin concentration of 8mg/L. Thus while the strains were not stable they merely required the presence of telithromycin to activate the resistance mechanism as the concentration required to increase the revertants telithromycin MIC was lower than that of M IV. The frequency of mutants was also very high at 1 per 10. Therefore, in order for *S. pneumoniae* with a *mefA* gene to maintain their telithromycin MIC the selective pressure must be applied. This is not true for those strains that contained the *ermB* gene or do not have either gene. Once resistance or elevated telithromycin MICs had been achieved in these strains, they remained stable whether or not there was selective pressure.

5.4 Discussion

The findings of the mutation study suggest that in order for telithromycin resistance to occur in *S. pneumoniae* either an *ermB* or *mefA* gene must be present. The mutants generated from a strain with the *ermB* gene were stable whereas the mutants from a *mefA* positive strain were not (table 12). However, the *mefA* revertants only required the presence of telithromycin to return to the highest telithromycin MIC once more. They did not return to the telithromycin MIC of 8mg/L by stepwise selection but merely in one step. Strain NCTC 13593 did not have either the *ermB* or *mefA* gene. The highest telithromycin MIC of any NCTC 13593 mutant was 0.5mg/L (table 10), which is still sensitive under the NCCLS breakpoint of 1mg/L. The MIC of the final generation NCTC 13593 mutants was greater than 20-fold higher than that of the parent NCTC 13593. While this is still sensitive it is a large increase in telithromycin MIC.

The frequency of mutant generation was greatest for the NCTC 13593 mutants (table 11). Although this did not produce telithromycin resistance it must be noted that the final generation were mutated at high frequency. If these were to acquire another advantage over telithromycin then there would be a large population base that could potentially become telithromycin resistant. With regard to the *ermB* generated mutants there is a large difference in the number of mutants formed from parent to first generation in comparison to first generation to second generation (table 11). This indicates that another mechanism has probably been created or acquired by the second generation mutants. For the *mefA* mutants the mutation

frequencies did not vary greatly from one generation to the next (table 11). Thus there was probably one mechanism of resistance at work that was refined and improved from one generation to the next as the concentration of telithromycin increased.

Overall these results should be taken as a warning that when *S. pneumoniae* is exposed to telithromycin *in vitro* the MIC will increase markedly. This is more important when the strains are already resistant to macrolides as the potential for the propagation of telithromycin resistant strains is increased. If telithromycin resistance can be generated *in vitro* with such ease, it is probable that telithromycin resistant *S. pneumoniae* will emerge in the community when the drug is released.

Chapter 6

Macrolide resistance genes *ermB* and *mefA*

6.1 Introduction

Macrolide resistance is an ever-increasing problem in *Streptococcus pneumoniae*. The two main known mechanisms of resistance to macrolides in *S. pneumoniae* are target modification and efflux. Target modification is mediated by the *ermB* gene, which encodes a 23S rRNA methylase. Methylation results in the *S. pneumoniae* being resistant to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics (Leclercq & Courvalin, 1991). Erm methylase synthesis can be inducible or constitutive. Bacteria resistant to erythromycin due to methylation are also resistant to azithromycin and clarithromycin (Nagai *et al.*, 2002). The *mefA* gene encodes an efflux pump, which confers resistance to 14 and 15 membered macrolides only. Therefore, strains containing the *mefA* gene are sensitive to lincosamides and streptogramin B antibiotics (Sutcliffe *et al.*, 1996b; Tait-Kamradt *et al.*, 1997; Roberts *et al.*, 1999). Recently another methylase gene, *ermA* was identified in *S. pneumoniae*. The strains containing the *ermA* were erythromycin resistant or intermediate, inducibly clindamycin resistant and streptogramin sensitive (Syrogiannopoulos *et al.*, 2001; Nagai *et al.*, 2002).

Telithromycin is a semi synthetic 14-membered-ring macrolide derivative, which is characterised as having a 3-keto function instead of an L-cladinose moiety and a C11-C12 carbamate link (Bryskier, 1998). These features are believed to enhance the telithromycin performance. It has been shown to be active against macrolide sensitive and resistant *S. pneumoniae* (Hamilton-Miller & Shah, 1998; Giovanetti *et al.*, 2000; Jalava *et al.*, 2001).

The aim of this study was to investigate the influence of the *ermB* and *mefA* genes in *S. pneumoniae* parent strains and mutants of varying telithromycin sensitivities. This involved screening the strains for the presence of the genes and then further molecular experiments to test if any mutations were present in these genes that could account for the decreased telithromycin susceptibilities.

6.2 Materials and Methods

6.2.1 Bacterial strains

Strains were selected as representative telithromycin derived mutants from each parent strain as shown in table 13.

Table 13. Representative telithromycin resistant mutants.

Parent	NCTC (0.016mg/L)	02J1095 (0.06mg/L)	02J1175 (0.5mg/L)
Generation 1	N I 1 (0.016mg/L)	J II 1 (1mg/L)	M I 3 (2mg/L)
	N I 7 (0.032mg/L)		M I 5 (2mg/L)
Generation 2	N II 7 (0.12mg/L)	J III 1 (4mg/L)	M II 15 (4mg/L)
		J III 8 (>32mg/L)	
Generation 3	N III 1(0.25mg/L)		M III 3 (8mg/L)
Generation 4			M IV (8mg/L)

6.2.2 Isolation of Chromosomal DNA

The strains were grown on Columbia agar supplemented with 5% defibrinated horse blood and the appropriate telithromycin concentration overnight. The parent strains and the NCTC mutants were inoculated onto telithromycin-free plates. Colonies were emulsified in 200 μL of MilliQ water in an eppendorf tube. They were boiled for 10 to 15 minutes in a boiling waterbath. The supernatant containing the extracted DNA was used as the DNA template in further experiments.

6.2.3 PCR conditions

The PCR conditions and the primers for the detection and amplification of the *ermB* and *mefA* genes were as previously described (Sutcliffe *et al.*, 1996b) and are described in table 14. The primers were supplied by MWG Biotech AG (Germany) and had been HPSF purified. The PCR reagents were obtained from Promega UK.

6.2.4 Primers

Table 14. PCR primers for *ermB* and *mefA* amplification.

Primer Name	Sequence 5' to 3'
ermB forward	GAA AA(AG) GTA CTC AAC CAA ATA
ermB reverse	AGT AA(CT) GGT ACT TAA ATT GTT TAC
mefA forward	AGT ATC ATT AAT CAC TAG TGC
mefA reverse	TTC TTC TGG TAC TAA AAG TGG

The reaction mixture for the amplification of the *ermB* gene consisted of 1μL of Taq DNA polymerase (5U/μL), 10μL of 10 x reaction buffer (50mM Tris-HCl (pH 8.0 @ 25°C), 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 1% Triton® X-100), 1μL of 100mM dNTP mix (0.25μL of each dNTP), 8μL of $MgCl_2$ (25mM), 1μL of primer mix (forward 13pmole/μL, reverse 25pmole/μL), 5μL of DNA template and 74μL of sterile MilliQ water. The Taq DNA polymerase was stored at -20°C and added to the mixture last. The reaction mixture was vortexed briefly and added to the PCR cycler. The PCR was performed in a Techne Thermal Cycler (Techne Cambridge Ltd., UK) under the conditions indicated in table 15.

Table 15. The PCR cycling conditions of *ermB* and *mefA*.

Number of cycles	Temperature (°C)	Time (minutes)
1	93	3
35	93	1
	52	1
	72	1
1	72	5

The positive control 02J1095 and a negative control, consisting of the PCR reaction mixture with 5μL of sterile MilliQ water instead of DNA template, were included in each PCR run.

For the *mefA* amplification the reaction mixture was the same as that of the *ermB* PCR mixture except for the $MgCl_2$ concentration, the primers and the amount of MilliQ water. In the case of the *mefA* the PCR mixture contained 16μL of $MgCl_2$ (25mM), 1μL of *mefA* primer mix (forward 17 pmol/μL and reverse 9 pmol/μL) and 66μL of sterile MilliQ water. The PCR cycling parameters were the same as those for the *ermB* amplification as shown in table 15. The positive control 02J1175 and a negative control were included in each PCR run.

The PCR samples were kept at 4°C until they were run on an agarose gel. If they were to be kept longer than one day, they were stored at -20°C.

6.2.5 Agarose Gel Electrophoresis

Two grams of electrophoresis grade agarose (Gibco BRL, Life Technologies, Paisley) was added to 100mL of 1 x TAE (10 x TAE: 48.4g Tris, 11.4mL glacial acetic acid, 20.0mL EDTA (pH 8.0) and 500mls of sterile distilled water, diluted 1 in 10 with sterile distilled water to produce 1 x TAE). The solution was heated in a microwave with swirling every 30 seconds until the agarose was dissolved. This was allowed to cool and then poured into the electrophoresis tank with the appropriate comb to a depth of about 2cm. The tank was transferred to the fridge to set before the comb was removed.

The solidified gel was submerged in 1 x TAE buffer. Ten micoliters of PCR product was mixed with 2μL of blue/orange 6 x loading dye (15% Ficoll®400, 0.03% Bromophenol Blue, 0.03% Xylene Cyanol FF, 0.4% Orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA). The mixture was then pipetted into a well in the gel. For the *ermB* and *mefA* PCR product gels, a 5μL of 100 base pair ladder and 1μL of 6 x loading dye mixture was run in 1 well of each gel. The electrophoresis was performed at 100 volts for 35 to 40 minutes. This allowed the ladder to separate and the PCR products to move down the gel.

The gel was immersed in ethidium bromide solution (20μL of a 10mg/mL solutions added to 200mLs of distilled water) for approximately 20 minutes. If the gel was not

sufficiently stained it was reimmersed in ethidium bromide solution. The DNA in the gel was visualised using Biorad imaging and diversity database computer software.

6.2.6 Purification and Quantification of PCR Products

The PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. The purified DNA was eluted in 30μL of sterile MilliQ water into an eppendorf tube. Two microliters of the purified DNA was run with blue/orange 6 x loading dye in a 2% agarose gel as previously described. In order to ascertain the quantity of DNA in the sample 4μL of 1Kb ladder or λ DNA/*Hin*d III marker was also run on the same gel. The gel was run, stained and visualised in the same way as the PCR product gels. The amount of DNA in the sample was quantified by comparing the brightness of the sample band with the bands of the 1Kb ladder or λ DNA/*Hin*d III marker.

6.2.7 Automated DNA sequencing

The primers used for the PCR amplification were also used for the DNA sequencing. Each primer was diluted in sterile MilliQ water to a final concentration of 3 pmol/μL for each sample. The DNA concentration of each sample was between 30 and 90ng. DNASHEF Technologies, Edinburgh, performed the sequencing. The DNA sequence was determined by the chain termination method (Sanger *et al.*, 1977). Individual

PCR fragments were set up in the Ready Reaction Format for fluorescence based on the dideoxy cycle sequencing (PE Applied Systems, UK).

The sequences were compared to previously published sequences of *S. pneumoniae* *ermB* and *mefA* genes using BLAST online search facility (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The *ermB* gene comparator was accession number X52632 and the *mefA* gene comparator was accession number U83667.

6.2.8 Efflux pump inhibition

In order to investigate the effect of efflux pumps on telithromycin resistance the parent strains 02J1095 and 02J1175 and the mutant strains M I 2, M I 5, M II 15, M III 3, M IV, J II 1, J III 1 and J III 8 were inoculated onto blood agar plates containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, UK). CCCP is a proton motive force inhibitor, which inhibits the *mefA* efflux pump (Sutcliffe *et al.*, 1996a).

Each of the strains were inoculated onto blood agar plates with a fixed concentration of CCCP at 10mg/L or 100μM per plate and varying concentrations of erythromycin or telithromycin and onto blood agar plates with varying concentrations of erythromycin or telithromycin and no CCCP, using a Denley Multipoint inoculator. The isolates were inoculated at a concentration of either 10^5 or 10^6 cfu per spot and incubated in 5% CO_2 overnight.

6.3 Results

The parent strain 02J1095 and the telithromycin derived mutants J II 1, J III 1 and J III 8 were all tested for the presence of the *ermB* gene. A 639 bp product was expected and this was amplified from each of the strains as shown in figure 12. The NCTC 13593 strain and the representative mutants were also investigated for the presence of *ermB* genes. No *ermB* genes were found in any of these strains as indicated in figure 13. The positive control included in each run did result in a PCR product corresponding to 639 bp each time the experiment was performed.

6.3.1 *ermB* Gels

Figure 12. *ermB* gene PCR gel of 02J1095 and representative mutants.

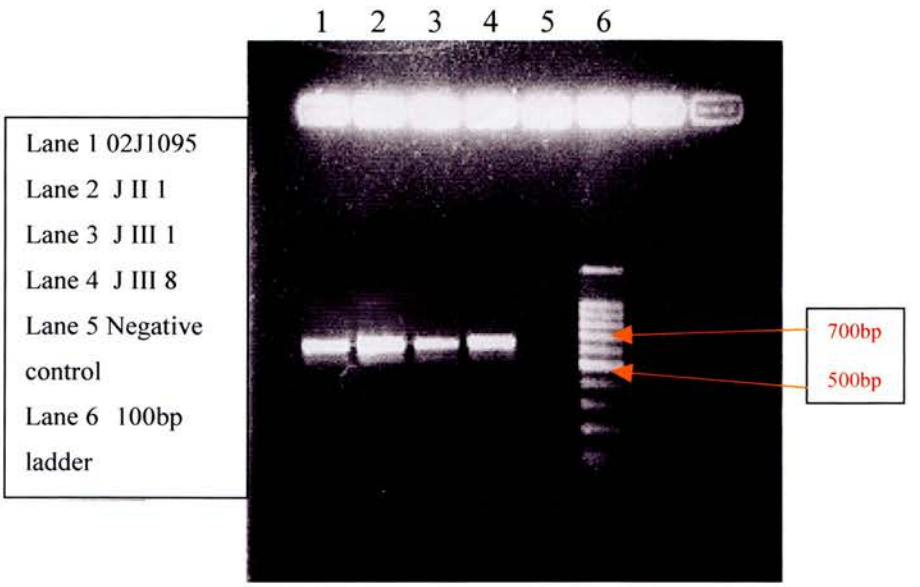
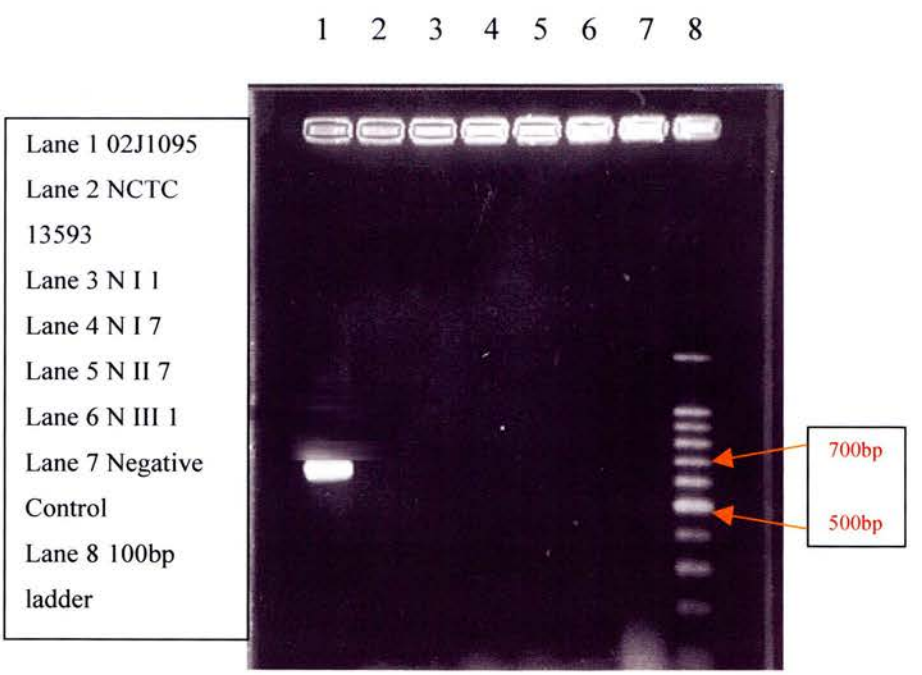


Figure 13. *ermB* gene PCR gel of 02J1095 positive control, NCTC 13593 and its representative mutants.



The 02J1175 parent and its representative mutants were screened for the *mefA* gene. The corresponding 348 bp product was amplified from each strain, as shown in figure 14. The NCTC 13593 parent and mutants were also screened for the *mefA* gene. No products were amplified from any of these strains, as indicated in figure 15. A 348 bp product was amplified from the positive control every time.

6.3.2 *mefA* Gels

Figure 14. *mefA* gene PCR gel of 02J1175 and representative mutants.

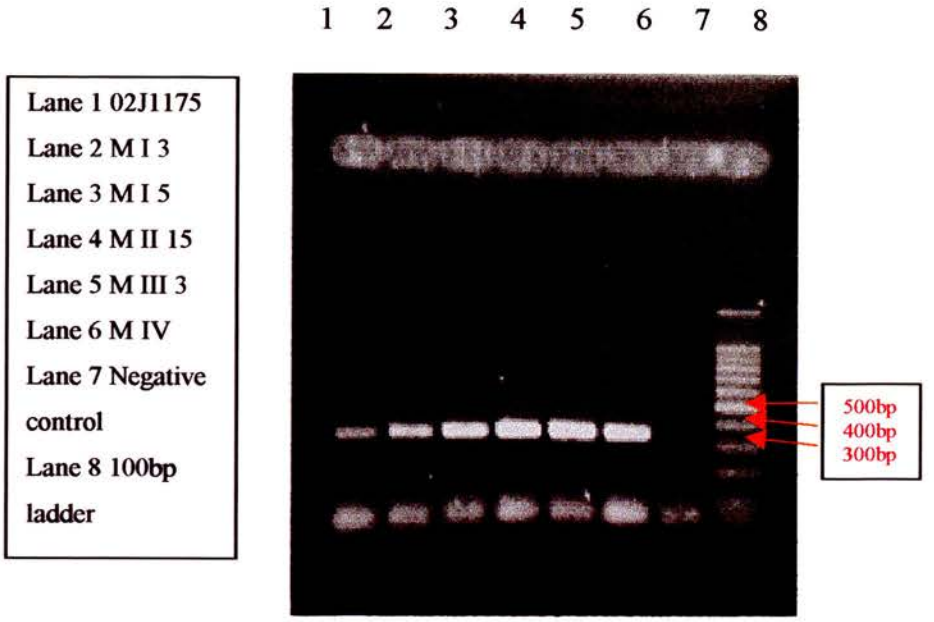
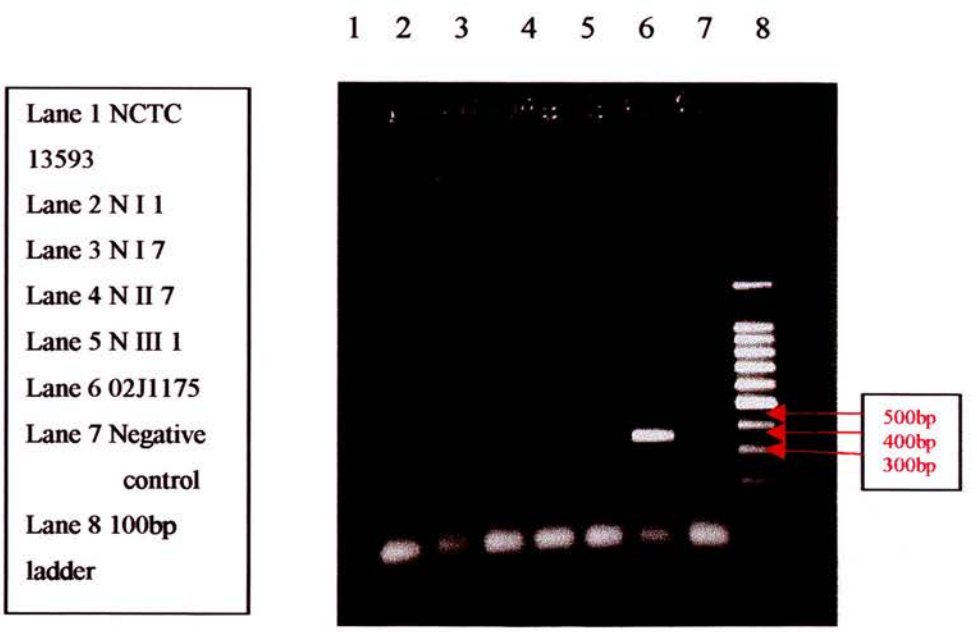


Figure 15. *mefA* gene PCR gel of 02J1175 positive control, NCTC 13593 and its representative mutants.



The *ermB* gene of each of the parent, 02J1095, and its mutants were sequenced. There were no changes in nucleotide sequence between the parent and any of the mutants tested. Even though the variation in telithromycin MIC was greater than 600-fold. There was however variation in each of the sequenced *ermB* genes and the *ermB* sequence of *S. pneumoniae* deposited in the NCBI website. The parent and the mutants all had the same mutations and are listed in table 16. Three mutations resulted in amino acid changes and one did not.

Table 16. *ermB* gene mutations.

Nucleotide change	Amino acid change
C to T	None
T to C	I to T
G to A	S to N
A to G	H to R

As all of these changes were present in all of the strains, including the telithromycin sensitive strains, they do not appear to be associated with increased telithromycin MIC.

6.3.3 *ermB* gene sequence and amino acid translation

gtttacgaaattggaacaggtaaagggcatttaacgacgaaactg

V Y E I G T G K G H L T T K L

gctaaaataagtaaacaggtaacgtctattgaattagacagtcat

A K I S K Q V T S I E L D S H

ctattcaacttatcgtcagaaaaattaaaactgaatactcgtgtc

L F N L S S E K L K L N T R V

actttaattcaccaagatattctacagtttcaattccctaacaaa

T L I H Q D I L Q F Q F P N K

cagaggtataaaattgttgggaatattccttaccatttaagcaca

Q R Y K I V G N I P Y H L S T

caaattattaaaaaagtggtttttgaaagccgtgcgtctgacatc

Q I I K K V V F E S R A S D I

tatctgattgttgaagaaggattctacaagcgtaccttggatatt

Y L I V E E G F Y K R T L D I

caccgaacactagggttgctcttgcacactcaagtctcgattcag

H R T L G L L L H T Q V S I Q

caattgcttaagctgccagcggaatgctttcatcctaaaccaaaa

Q L L K L P A E C F H P K P K

gtaaacagtgtcttaataaaacttacccgccataccacagatgtt

V N S V L I K L T R H T T D V

ccagataaatattggaagctatatacgtactttgtttcaaaatgg

P D K Y W K L Y T Y F V S K W

gtcaatcgagaatatcgtcaactgtttactaaaaatcagtttcat

V N R E Y R Q L F T K N Q F H

caagcaatgaaacacgccaaagtaaacaat

Q A M K H A K V N N

The sequences of the portion of the *mefA* gene of the parent 02J1175 and its mutants were identical to each other and the *mefA* gene sequence in the NCBI website. The

mefA gene in the NCBI website was isolated from 02J1175. The 02J1175 strain used in these experiments was therefore identical to the NCBI strain. The mutants however had elevated telithromycin MICs in comparison to its parent, which cannot be attributed to changes in the *mefA* gene as none were found.

6.3.4 *mefA* sequence and translation (portion of total *mefA* gene)

```
gtatcattaatcactagtgccatcctgcaaatggcgattattttt
 V  S  L  I  T  S  A  I  L  Q  M  A  I  I  F
taccttacagaaaaaacaggatctgcgatggtcttgtctatggct
 Y  L  T  E  K  T  G  S  A  M  V  L  S  M  A
tcattagtaggtttttaccctatgcgattttgggacctgccatt
 S  L  V  G  F  L  P  Y  A  I  L  G  P  A  I
ggtgtgctagtggatcgtcatgataggaagaagataatgattggt
 G  V  L  V  D  R  H  D  R  K  K  I  M  I  G
gccgatttaattatcgcagcagctggtgcagtgcttgctattgtt
 A  D  L  I  I  A  A  A  G  A  V  L  A  I  V
gcattctgtatggagctacctgtctggatgattatgatagtattg
 A  F  C  M  E  L  P  V  W  M  I  M  I  V  L
tttatccgtagcattggaacagcttttcataccccagcactcaat
 F  I  R  S  I  G  T  A  F  H  T  P  A  L  N
gcggttacaccacttttagtaccagaagaa
 A  V  T  P  L  L  V  P  E  E
```

6.3.5 Efflux pump results

No growth was observed on any of the plates containing CCCP including control plates with no antimicrobial agent. All of the isolates grew on the plates without CCCP and had the same MICs as previously recorded. As none of the isolates survived in the presence of CCCP it is impossible to say what the influence of CCCP inhibited efflux pumps has on telithromycin MIC as the concentration required to inhibit the pumps also inhibited the bacterial growth.

6.4 Discussion

The *ermB* and *mefA* genes have been widely accepted as the main causes of macrolide resistance in *S. pneumoniae*. The ketolide telithromycin is an erythromycin derivative and so has a similar mechanism of action to the macrolides. Thus, it could be expected that resistance to telithromycin could result from mutations or adaptations of the resistance mechanisms already widespread to the macrolides. However, the *ermB* methylase and the *mefA* efflux mechanisms are not capable of causing resistance to telithromycin. Also, from the results presented in this study *S. pneumoniae* has not been able to alter these genes to overcome the antibacterial effect of telithromycin. *Streptococcus pneumoniae* has managed to adapt to become telithromycin resistant and highly resistant to telithromycin. The mechanism used however is not an alteration of methylase production controlled by

the *ermB* gene or adapting the efflux mechanism associated with the portion of the *mefA* gene amplified and sequenced. The NCTC 13593 parent and its mutants were *ermB* and *mefA* negative as expected as they were all macrolide sensitive.

The mutations identified in the *ermB* genes were present in all of the strains. The lowest telithromycin MIC of strains with the mutated *ermB* gene was 0.06mg/L, which was telithromycin sensitive. So even though there were three amino acid changes in the methylase gene sequence they do not appear to affect the ErmB methylase or its activity.

Chapter 7

23S rRNA Gene Mutation

7.1 Introduction

Erythromycin interacts with the domains II and V of the 23S rRNA of *Streptococcus pneumoniae* (Menninger & Otto, 1982; Brisson-Noël *et al.*, 1988, Leclercq & Courvalin, 1991; Weisblum, 1995a; Garza-Ramos *et al.*, 2001; Zhanel *et al.*, 2001). The central loop of domain V is known as the peptidyl transferase region of the ribosome. Mutations in the ribosome have previously been shown to cause macrolide resistance in bacteria (Meier *et al.*, 1994; Versalovic *et al.*, 1996; Ross *et al.*, 1997; Vester & Douthwaite, 2001). Tait-Kamradt *et al.*, (2000b) described the first mutations in the domain V of the 23S rRNA in *S. pneumoniae* which resulted in macrolide resistance. These mutations were identified in laboratory derived mutants. They also determined that *S. pneumoniae* has four copies of the gene encoding the 23S rRNA. The number of genes with a mutation in the peptidyl transferase region of the 23S rRNA varied from one to four. The *S. pneumoniae* with mutated 23S rRNA did not contain either the *ermB* or *mefA* genes. Two of the ribosomal mutations located by Tait-Kamradt *et al.*, (2000b) have since also been identified in macrolide resistant clinical isolates (Pihlajamaki *et al.*, 2002).

There are two different systems of numbering nucleotides in a gene or sequence. The most common method is to use the nucleotide number associated with the position of that nucleotide in the *Escherichia coli* sequence. The second nucleotide numbering scheme originates from its position in the bacteria of study, in this case, in the *S. pneumoniae* genome. The numbers are within a few positions of each other e.g. nucleotide number 2058 in *E. coli* is number 2060 in *S. pneumoniae*. Unless specifically stated *E. coli* numbering is assumed.

The sites of mutation within the domain V were initially 2058, 2059 and 2611. Another mutation at 2062 was later also classified in macrolide resistant *S. pneumoniae* (Depardieu & Courvalin, 2001). Mutations U2609C and A2058G together, lead to telithromycin resistance in *E. coli* (Garza-Ramos *et al.*, 2001). This phenomenon has not been associated with telithromycin resistance in *S. pneumoniae* as no strain with such mutations has been generated or clinically isolated.

Xiong *et al* (1999), Hansen *et al.*, (1999) and Douthwaite *et al.*, (2000) have all highlighted the interaction of erythromycin and telithromycin with the hairpin 35 of domain II of the 23S rRNA of *E. coli*. Telithromycin had a strong affinity for hairpin 35 particularly at adenine 752. Recently, Canu *et al.*, (2002) revealed that a deletion of one adenine in the series of four located between 749 and 752 in the domain II of *S. pneumoniae* lead to a 500-fold increase in the telithromycin MIC of the strain. This increase resulted in the strain being telithromycin resistant. The mutant was a laboratory-generated strain selected on clarithromycin.

As such mutations have been associated with macrolide resistance and later ketolide resistance it was logical to assume that the telithromycin resistant generated mutants created from 02J1095 and 02J1175 could also have mutations in either domain II or V of the 23S rRNA. Therefore, we decided to look for mutations in any of the four 23S rRNA genes in domains II and V which could account for the telithromycin resistance.

7.2 Materials and Methods

7.2.1 Bacterial strains

The parents 02J1095 and 02J1175 and the representative mutants J II 1, J III 1, J III 8, M I 3, M II 15, M III 3 and M IV, previously described in tables 8, 9 and 13, were investigated in this study.

7.2.2 PCR

In order to detect mutations in the peptidyl transferase region of the 23S rRNA all four contigs of the domain V were amplified using four downstream (DS) primers as described by Tait-Kamradt *et al.*, (2000b) and in table 17. The Domain II portion of 23S rRNA of J III 8 and M IV were amplified using the 23 S 5′ primers described by Tait-Kamradt *et al.*, (2000b) as shown in table 17. Problems were encountered

obtaining reproducible sequence data for the nucleotide section from 2350 to 2650 (*S. pneumoniae* numbering) of the four domain V genes. As a result primers were designed using the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi to amplify this inner portion seperately see table 17 for details.

DNA was isolated from each strain as described in the previous chapter and used for the domain V and domain II gene amplifications. Purified domain V PCR products were used as the DNA template for the amplification of the inner portion of the genes.

Table 17. Primers for the PCR amplification of the peptidyl transferase regions of 23S rRNA.

Primer name	Sequence 5′ to 3′
Forward 23S 3'	CGG CGG CCG TAA CTA TAA CG
Reverse	
DS 18	GCC AGC TGA GCT ACA CCG CC
DS 23	TAC ACA CTC ACA TAT CTC TG
DS 30	TTT TAC CAC TAA ACT ACA CC
DS 91	TAC CAA CTG AGC TAT GGC GG
Inner primers	
Forward	GTT CCC TCA GAA TGG TTG GA
Reverse	CAT AGC TAC CCA GCG ATG C
Domain II	
Forward	GGT TAA GTT AAT AAG GGC GC
Reverse	TTT CGA CTA CGG ATC TTA GC

7.2.3 PCR reagents

The PCR reaction contents were the same as the PCR mixture of *ermB* except for the modifications described in table 18.

Table 18. 23S rRNA PCR reaction mixtures.

PCR product	$MgCl_2$ concentration (mM)	Sterile MilliQ water (μL)	Primer concentration Forward/Reverse (pmol/μL)
DS 18	2	74	66/47
DS 23	2	74	66/81
DS 30	2	74	66/82
DS 91	3	70	66/21
Inner segment	2	74	91/79
Domain II	4	66	29/39

The setup of the PCR reaction mixture and the storage of the PCR products were the same for that of the *ermB* PCR products. The PCRs were also performed in a Techne Thermal Cycler under the following conditions described in table 19.

Table 19. 23S rRNA PCR cycle parameters

PCR product	Number of cycles	Temperature (°C)	Time (minutes)
DS 18	1	94	3
DS 23	35	94	1
	35	44	1
	35	72	1
	1	72	10
DS 30	1	94	3
DS 91	35	94	1
Inner section	35	54	1
Domain II	35	72	1
	1	72	10

7.2.4 Agarose Gel Electrophoresis

The PCR products were run on 2% agarose gels, stained and visualised as described in the previous chapter.

7.2.5 Purification and Quantification of PCR Products

All PCR products except DS 18 were purified as previously described using a QIAquick PCR purification kit. The amplification of the DS 18 gene segment resulted in two PCR products represented by two bands on the gel, which can be seen in figure 16. The DS 18 PCR products were run on 2% low melting temperature (LMT) agarose (Nusieve) to enable isolation of the DNA from the 2000 nucleotide

band. The LMT agarose was poured into the electrophoresis gel tank until the agarose filled the tank up to the top of the comb wells and was allowed to solidify in the fridge. The LMT agarose gels took longer to set than the standard agarose gels. The DS 18 PCR products (90 μL) were mixed with 6 x loading dye and pipetted into the gel wells. A 1kb ladder was included in each gel. The gel was run at 100 V for 40 minutes in 1 x TAE. The gel was stained for approximately 30 minutes in ethidium bromide solution. The bands were visualised on a UV transilluminator (UV products). The band at 2000 bp was cut out of the gel and purified using a QIAquick gel extraction kit (Qiagen Ltd., Crawley, UK). The purified product was eluted in 30 μL of sterile MilliQ water. All purified products were run on a 2% agarose gel and the DNA quantified as previously described.

7.2.6 Automated DNA Sequencing

The PCR products of J III 8 and M IV for each of the amplified portions of the domain V and domain II segments of the 23S rRNA genes were sequenced by DNASHEF Technologies, Edinburgh. Each of the sequences were analysed for mutations by comparison with the 23S rRNA sequence of *S. pneumoniae* TIGR 4 (http://www.tigr.org/tigr- scripts/CMR2/rna.spl?db=bsp). The sequences of J III 8 or M IV and the nucleotide sequence of 23S rRNA of TIGR 4 were entered into the BLAST pairwise analysis program to align the nucleotides.

7.3 Results

Figure 16. DS 18 23S rRNA PCR products gel.

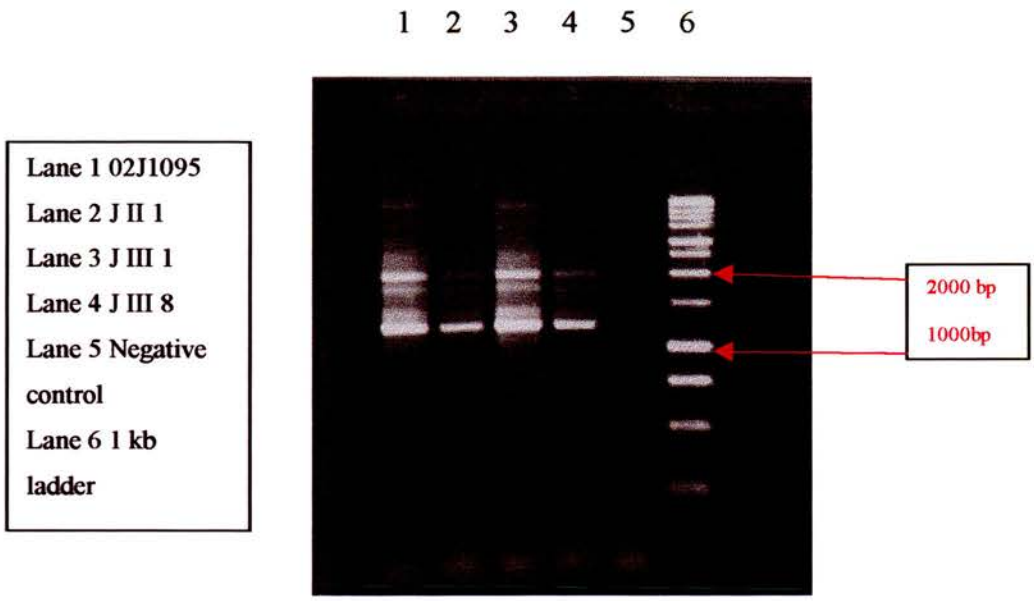


Figure 17. DS 23 23S rRNA PCR products gel.

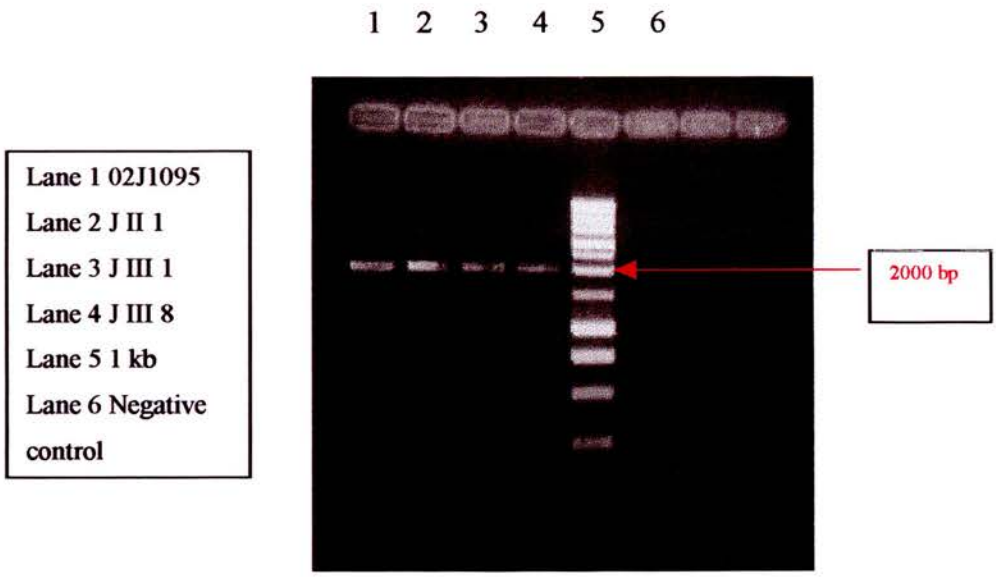
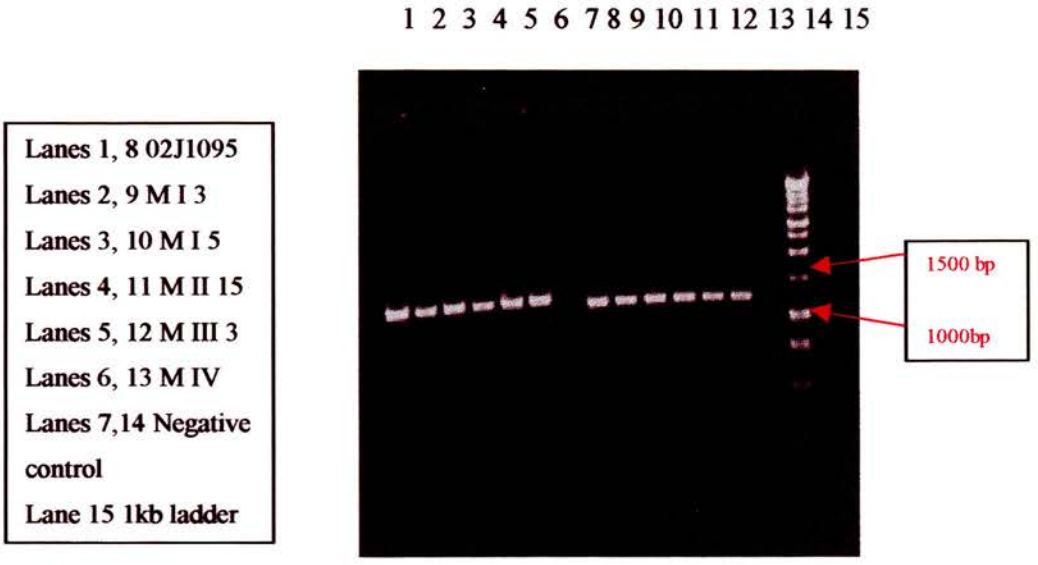


Figure 18. DS 30 and DS 91 23S rRNA PCR products gel.



Lanes 1 to 7 contain DS 30 23S rRNA PCR products and lanes 8 to 14 contain DS 91 23S rRNA PCR products.

Figure 19. Inner section of 23S rRNA for DS 18 and DS 23 PCR products

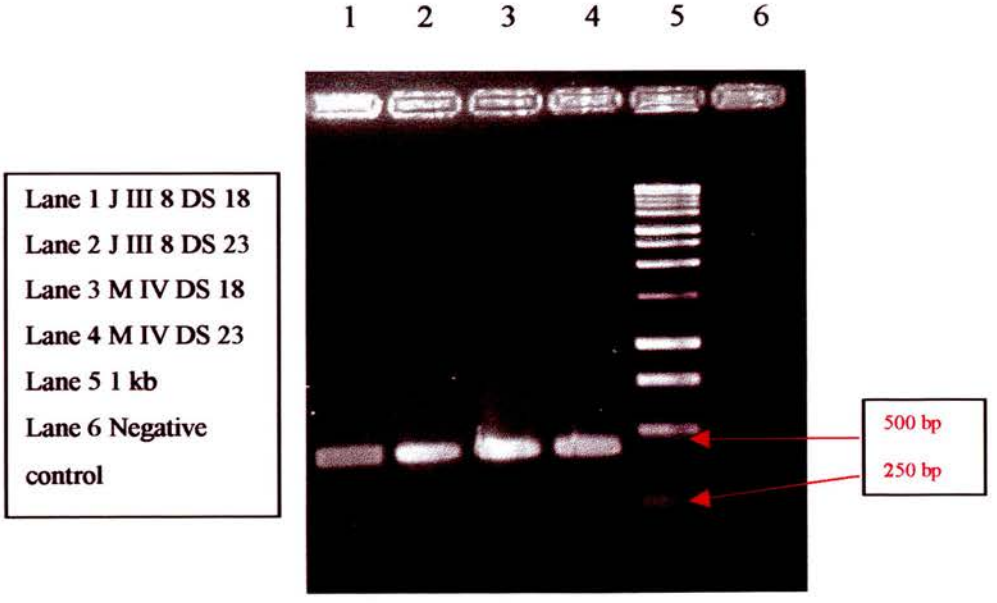
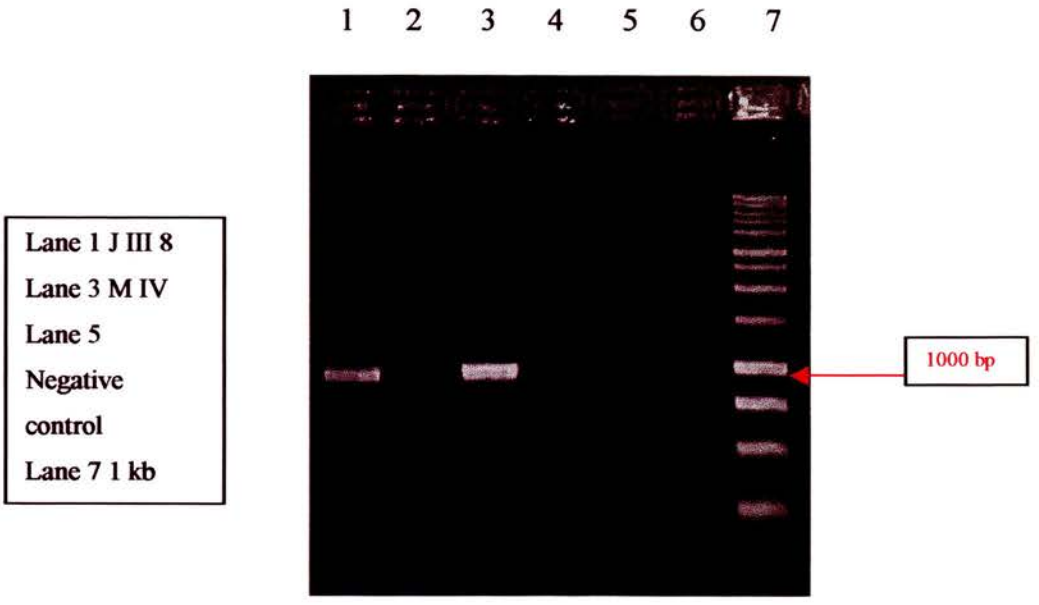


Figure 20. 23S rRNA Domain II PCR products



The primers used to amplify the four copies of the 23S rRNA gene each had unique reverse downstream primers as shown in table 20. The downstream reverse primers were designed such that they amplified a segment outside of the 23S rRNA gene, which was unique to that copy. This resulted in four gene segments, each of a different size described in table 20. The use of these primers enabled the peptidyl transferase region from all four alleles of 23S rRNA to be separately amplified, as shown in figures 16 to 18, and purified for DNA sequencing. Downstream (DS) 18 PCR products consisted of the 23S rRNA region from 1899bp to 1000bp past the end of the 23S rRNA gene. The resulting bands of 2000 bps are shown figure 16. The DS 23 PCR product, in figure 17, consisted of approximately the same distance past the end of the 23S rRNA but the gene amplified was not the same as that of DS 18. The DS 30 and DS 91 PCR products, figure 18, each amplified from 1899bp to 300bp and 200bp respectively past the end of the 23S rRNA genes.

Table 20. Position of the downstream primers in relation to the 23S rRNA genes

Downstream gene	23S rRNA gene (bp)	Number of nucleotides after the 23S rRNA gene
DS 18	1899 to 2902	+ 997
DS 23	1899 to 2902	+ 997
DS 30	1899 to 2902	+ 297
DS 91	1899 to 2902	+ 197

The downstream 23S rRNA genes were amplified from each of the parents, 02J1095 and 02J1175, and also the representative mutants as shown in figures 16 to 18. Only the four domain V genes of J III 8 and M IV were entirely sequenced. The sequences from the DS 30 and DS 91 genes were obtained using only the 23S rRNA and downstream primers and not the inner primers. The sequencing results of the DS 18 and DS 23 genes were incomplete using the downstream primers. The inner portion from 2350 to 2650 (*S. pneumoniae* numbering) contained many unknown nucleotides. The inner segments were separately amplified, which can be seen in figure 19, and sequenced for J III 8 and M IV. When the entire sequences of the four copies of the 23S rRNA domain V genes were obtained they were compared to the 23S rRNA gene sequences of TIGR 4. No nucleotide changes, insertions or deletions were located in any of the four copies of the 23S rRNA domain V genes of J III 8 or M IV.

The domain II portion of the 23S rRNA genes were also amplified as shown in figure 20, and sequenced for J III 8 and M IV using primers which amplified the first 1011 nucleotides of the 23S rRNA genes. This 1011 nucleotide portion of 23S rRNA contains the domain II region. Only one copy of the genes were sequenced.

Mutations would be shown by heterogeneity in the nucleotide sequence. The region of interest in domain II was hairpin 35, which stretched from nucleotide 734 to 762 (*E. coli* numbering). No changes, insertions or deletions were identified in this region or in the nucleotides surrounding this region of J III 8 and M IV in comparison to TIGR 4.

7.4 Discussion

Mutations in the domain V and domain II of the 23S rRNA genes of *S. pneumoniae* have previously been associated with increased macrolide and ketolide MICs (Tait-Kamradt *et al.*, 2000b; Canu *et al.*, 2002). The four genes of the domain V of the telithromycin resistant strains J III 8 and M IV contained no such mutation, neither did the genes encoding domain II of these strains. The domains V and II of the telithromycin resistant strains were identical to those of the telithromycin sensitive TIGR 4.

The changes in the domain V or domain II of the 23S rRNA that have previously been identified were in *ermB* and *mefA* negative strains. In these cases the mutations in the 23S rRNA alone were responsible for the macrolide resistance. Only a deletion in the domain II has previously been associated with telithromycin resistance in *S. pneumoniae*.

The results generated by this study question firstly the role of the mutations in domain V on telithromycin resistance in *S. pneumoniae*, secondly the importance of the mutation associated with telithromycin resistance in domain II and thirdly the association between the mutations in *E. coli* leading to telithromycin resistance in domain V and its implications for *S. pneumoniae*. The fact that J III 8 and M IV contained *ermB* and *mefA* genes respectively has been shown not to cause telithromycin resistance in these strains. However, it is uncertain if the presence of either one of these genes would prevent mutations in the 23S rRNA, as a mechanism of macrolide resistance already exists. In conclusion, telithromycin resistance in *S. pneumoniae* with either an *ermB* or *mefA* gene is not mediated by mutations in either the domain V or domain II regions of the 23S rRNA. Therefore, the binding sites of telithromycin, as presently known, were not mutated in order to prevent telithromycin binding to the 23S rRNA.

Chapter 8

L4 and L22 Gene Mutations

8.1 Introduction

The ribosome consists of both rRNA and ribosomal proteins. The way in which both interact together has not yet been fully elucidated. The L4 and L22 riboproteins are two such ribosomal proteins, which form part of the 23S rRNA region of the ribosome. The first reports of ribosomal structural changes in erythromycin resistant mutants of *E. coli* described ribosomal protein alterations of L4 and L22 (Wittman *et al.*, 1973; Arávélo *et al.*, 1988). Both L4 and L22 have been shown to interact directly with the 5′ portion of the 23S rRNA which contains the domain II region strongly associated with ketolide binding (Rohl & Nierhaus, 1982). Therefore, these proteins form an important part of the ribosomal region associated with the macrolide and ketolide ribosomal interaction and when mutated could also have a role in macrolide and ketolide resistance.

The first report of mutations in L4 associated with increased macrolide MICs in *S. pneumoniae* was published in August 2000 (Tait-Kamradt *et al.*, 2000b). The mutations consisted of a glycine to cysteine change at amino acid 69 and a 6 base-pair in-frame insertion between amino acid 67 and 68. The two mutations occurred in two different strains and neither resulted in erythromycin nor

telithromycin resistance. Clinical isolates of *S. pneumoniae* were soon discovered in Eastern Europe and Canada with mutations in the same conserved region of the L4 protein. These however, were macrolide and lincosamide resistant (Tait-Kamradt *et al.*, 2000a).

Mutations in L22 associated with decreased macrolide and ketolide activity have recently been located in laboratory derived *S. pneumoniae* mutants. The amino acid mutations were located at amino acid positions 83, 91, 93, 95 and 99 (Canu *et al.*, 2002). There have only been three clinical isolates of *S. pneumoniae* identified with an L22 mutation. This was a G95D amino acid substitution and was in combination with an A2059G mutation in all 4 copies of the 23S rRNA genes (Farrell *et al.*, 2002). These clinical strains were erythromycin resistant but telithromycin sensitive. None of the *S. pneumoniae* isolates with L4 or L22 mutations contained either *mefA* or *ermB* genes. The number of macrolide resistant *S. pneumoniae* strains, resistant due to either methylation by the *ermB* gene or efflux by the *mefA* gene is higher than those resistant because of ribosomal mutations (Farrell *et al.*, 2002).

As the mutants generated in this study did not contain mutations in the 23S rRNA genes I looked to the L4 and L22 ribosomal proteins for answers to the question of telithromycin resistance. The hypothesis was that mutations in both L4 and L22 have previously lead to increased telithromycin MICs in *S. pneumoniae* and so, if mutated, could cause an increase in telithromycin MICs.

8.2 Materials and Methods

8.2.1 Bacterial strains

The parent strains 02J1095 and 02J1175 and the mutants J III 8 and M IV were investigated for mutations in the L4 riboprotein .The L22 proteins of strains 02J1095, J II 1, J III 1, J III 4, J III 5, J III 6, J III 7, J III 8, J III 9, 02J1175 and M IV were also checked for mutations. These strains have previously been described in tables 8, 9 and 13.

8.2.2 PCR

The L4 and L22 genes of the bacterial strains were amplified using PCR. DNA was isolated from each strain as described in the previous chapters. The primers used to amplify the L4 and L22 genes were those of Tait-Kamradt *et al.*, (2000b) and are listed in table 21.

Table 21. Primers used to amplify the L4 and L22 genes.

Primer name	5′ to 3′
L4 forward	AAA TCA GCA GTT AAA GCT GG
L4 reverse	GAG CTT TCA GTG ATG ACA GG
L22 forward	GCA GAC GAC AAG AAA ACA CG
L22 reverse	ATT GGA TGT ACT TTT TGA CC

8.2.3 PCR reagents

The PCR reaction mixture consisted of the same contents as those for the *ermB* gene PCR mixture except for the primers listed in table 21. The L4 primers were 39pmol/μL for the forward primer and 75pmol/μL for the reverse primer. The L22 forward and reverse primer concentrations were 66pmol/μL and 44pmol/μL respectively.

8.2.4 PCR cycle parameters

The reaction mixture was set up in a Techne Thermal Cycler under the conditions described in table 22 for the L4 PCR amplification and table 23 for the L22 PCR amplification..

Table 22. L4 PCR cycle parameters.

Number of cycles	Temperature (°C)	Time (minutes)
1	94	3
35	94	1
35	54	1
35	72	1
1	72	10

Table 23. L22 PCR cycle parameters.

Number of cycles	Temperature (°C)	Time (minutes)
1	94	3
35	94	1
35	44	1
35	72	1
1	72	10

8.2.5 Agarose gel electrophoresis

The PCR products were run on 2% agarose gels stained and visualised as previously described.

8.2.6 Purification and Quantification of PCR Products

The L4 PCR products were purified as previously described using a QIAquick PCR purification kit. The amplification of the L22 gene resulted in two PCR products represented by two bands on the gel. The L22 PCR products were run on 2% low melting temperature agarose and purified in the same way as the DS 18 PCR products in the previous chapter.

8.2.7 Automated DNA Sequencing

The purified L4 and L22 genes were sequenced by DNASHEF technologies, Edinburgh. The nucleotide sequences of L4 and L22 genes were compared to previously published sequences of *S. pneumoniae* L4 and L22 genes using the BLAST online search facility (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The L4 genes were compared to the sequence of TIGR 4 accession number AE 007335.1 from nucleotide 7367 to 7990 and the L22 to nucleotide 9534 to 9878 of TIGR 4. The nucleotide sequences were also translated using the ExPaSy online translate tool at http://www.expasy.ch/tools/dna.html and the amino acid sequences were compared with those of L4 and L22 of TIGR 4.

8.2.8 Transformation

To access the role of the mutated L22 gene in telithromycin resistance the resistant alleles of the L22 gene of J III 8 were amplified and purified. Transformation of the mutated L22 gene into *Streptococcus pneumoniae* NCTC 13593 was attempted as previously described by Canu *et al* (2002).

Competent cells were prepared as follows: *Streptococcus pneumoniae* NCTC 13593 was inoculated into 10mL of CAT broth (1000mL sterile distilled water, 5g NaCl (Sigma), 1g yeast extract (Sigma), 5g Todd Hewitt broth (Oxoid), 10g enzymatic casein hydrolysate (Oxoid)) with 0.2% glucose (Sigma) and 15mM K_2HPO_4 (BDH

laboratories) (CAT I) and incubated in 5% CO_2 overnight. One hundred microliters of the overnight culture was inoculated into CAT I supplemented with 0.2% bovine serum albumin (Promega) and 1mM $CaCl_2$ (Sigma) (CAT II). The broth culture was incubated in 5% CO_2 overnight. The cells were centrifuged at 3900 rpm for 10 minutes in a Sorvall RT7 Plus centrifuge. The pellet was resuspended in 1mL of CAT II with 15% glycerol. The competent cells were split into 100 µL aliquots and stored at -70°C.

For transformation the cells were thawed on ice and 10µL was added to 10mL of CAT II, which had been adjusted to pH 7.8. The mixture was then incubated in 5% CO_2 at 37°C for 15 minutes. Ten microliters of purified L22 gene PCR product were added to 1mL of the culture and the reaction mixture was incubated in 5% CO_2 and 37°C for varying times of 1 hour, 2 hours, 3 hours, 3 hours 35 minutes, 5 hours, 5 hours 15 minutes and overnight. A negative control with 10µL of sterile milliQ water instead of purified PCR product was added with each run.

One hundred microliters of the resulting cultures were spread on Columbia agar plates supplemented with 5% defibrinated horse blood and telithromycin concentrations of 4mg/L. Control plates with no antibiotic were also included in each assay. All plates were incubated in 5% CO_2 for 48 hours.

8.3 Results

The L4 and L22 ribosomal protein genes of the isolates were amplified and the corresponding PCR bands at 720 bp and 420 bp were visualised on the agarose gels shown in figures 21, 22 and 23.

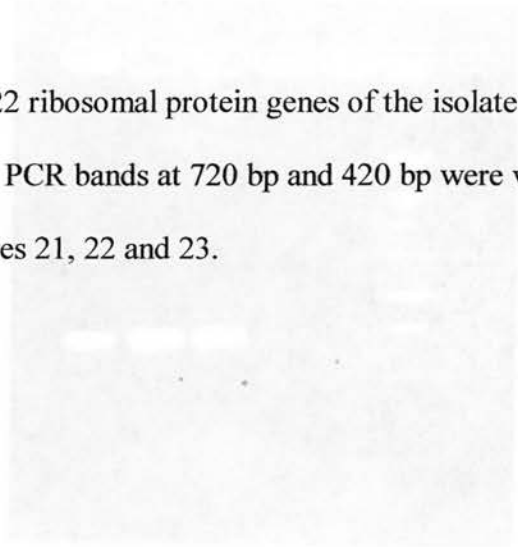


Figure 21. L4 PCR products gel

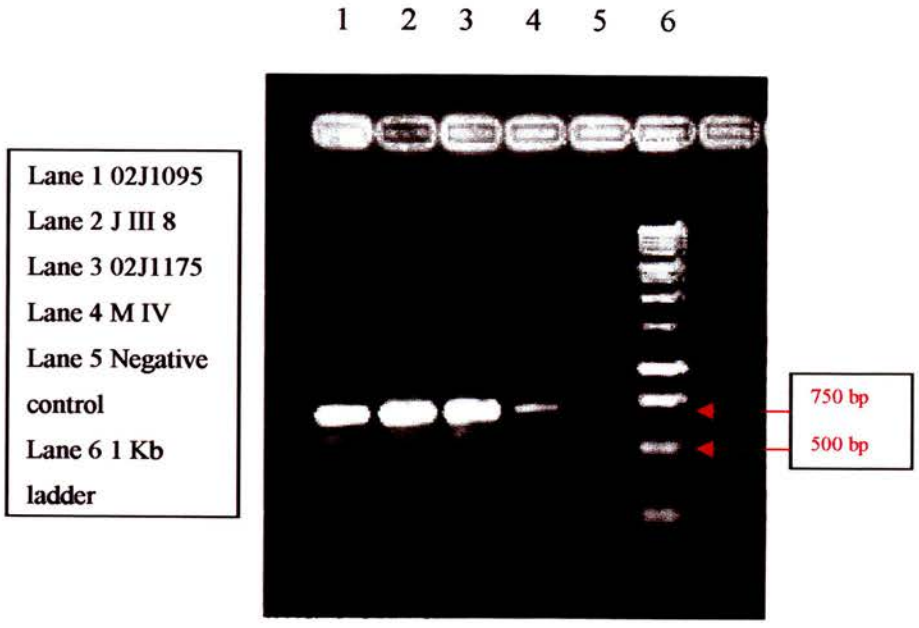


Figure 22. Purified L22 PCR products gel

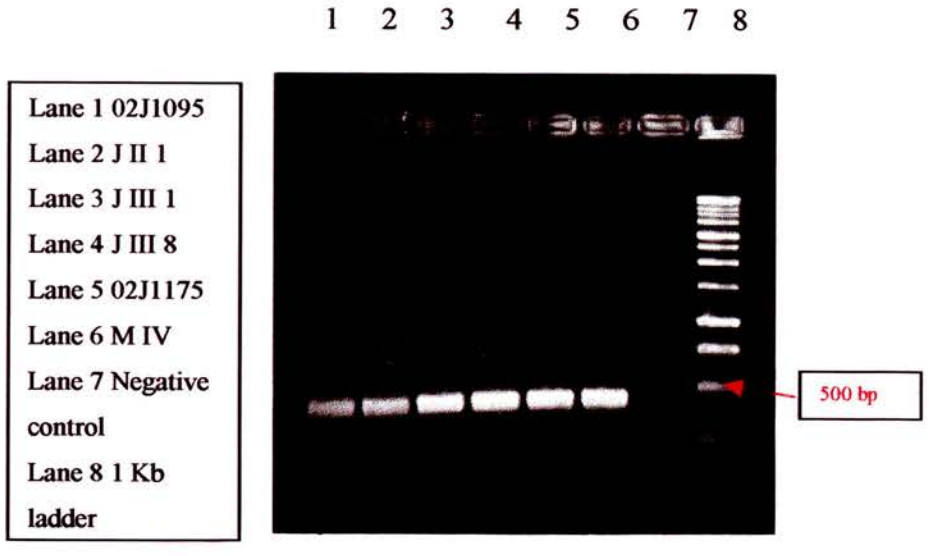
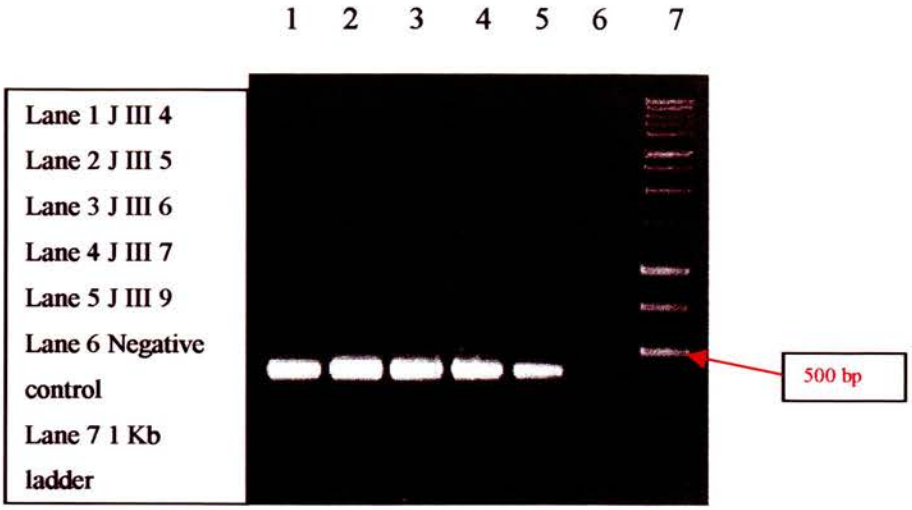


Figure 23. L22 PCR products gel.



Neither the nucleotide nor the amino acid sequences of the L4 riboproteins of 02J1095, J III 8, 02J1175 or M IV contained any mutations in comparison to those of TIGR 4. The L22 nucleotide and amino acid sequences of 02J1095, J II 1, J III 1, J III 4, J III 5, J III 6, J III 7, J III 9, 02J1175 and M IV were also the same as those of TIGR 4. The parent strains 02J1095, 02J1175 and J II 1 were erythromycin resistant but telithromycin sensitive, whereas J III 1 to J III 9 and M IV were macrolide and telithromycin resistant. A mutation in the sequence of the L22 of J III 8 at nucleotide position 9813 (*S. pneumoniae* numbering) from adenine to cytosine was the only mutation identified as shown in the amino acid sequence below. This altered the amino acid sequence also, by changing the lysine residue at amino acid 94 to glutamine. The L22 gene sequences of the other *ermB* positive mutants with telithromycin MICs between 1mg/L and greater then 32mg/L did not contain any mutation.

8.3.1 L22 gene sequence and amino acid translation

```
atggcagaaattacttcagctaaagcaatggctcgtacagtacgt
 M  A  E  I  T  S  A  K  A  M  A  R  T  V  R
gtttcacctcgtaaatcacgtcttgttcttgataacatccgtggt
 V  S  P  R  K  S  R  L  V  L  D  N  I  R  G
aaaagcgtagccgatgcaatcgcaatcttgacattcactccaaac
 K  S  V  A  D  A  I  A  I  L  T  F  T  P  N
aaagctgctgaaatcatcttgaaagttttgaactcagctgtagct
 K  A  A  E  I  I  L  K  V  L  N  S  A  V  A
aacgctgaaaacaactttggtttggataaagctaacttggtagta
 N  A  E  N  N  F  G  L  D  K  A  N  L  V  V
tctgaagcattcgcaaacgaaggaccaactatgaaacgtttccgt
 S  E  A  F  A  N  E  G  P  T  M  K  R  F  R
ccacgtgcgcaaggttcagcttcaccaatcaacaaacgtacagct
 P  R  A  Q  G  S  A  S  P  I  N  K  R  T  A
cacatcactgtagctgttgcagaaaaa
 H  I  T  V  A  V  A  E  K
```

This is the first incidence of a $_{94}$K to Q$_{94}$ change in L22 associated with telithromycin resistance. The mutation was only in the highly telithromycin resistant J III 8 strain. After this mutation was discovered further strains were investigated. The L22 genes of J III 4, J III 6, J III 7 and J III 9, with telithromycin MIC values of 16mg/L,

32mg/L, >32mg/L and >32mg/l respectively, were amplified as shown in figure 23, and sequenced. None of these strains contained any mutation in their L22 genes.

In the transformation experiments growth was observed at each time point on the non-selective plates with no telithromycin. However, no colonies were present on the transformant selective plates containing telithromycin from any of the time points. Therefore the effect of the L22 mutation alone on the development of telithromycin resistance could not be determined as the mutated L22 was not transformed into the telithromycin sensitive NCTC 13593 strain.

8.4 Discussion

The L4 and L22 proteins are both associated with a tunnel in the 50S ribosome subunit through which nascent peptides exit from the ribosome (Gabashvili *et al.*, 2001). Macrolides are a group of antibiotics that act in the vicinity of the peptidyltransferase centre and the entrance of the polypeptide tunnel. Arévälo *et al.*, (1988) suggested that erythromycin could physically block this tunnel. Mutations in either the L4 or L22 proteins could alter their conformations and prevent macrolide or ketolide binding to the rRNA (Gregory & Dahlberg, 1999). Both L4 and L22 mutations have been associated with erythromycin resistance (Weisblum, 1995a). Mutations in L4 and L22 have also been identified in *S. pneumoniae* with increased telithromycin MICs and erythromycin resistance both in laboratory derived mutants

and clinical isolates (Tait-Kamradt *et al.*, 2000a; Tait-Kamradt *et al.*, 2000b; Canu *et al.*, 2002; Farrell *et al.*, 2002; Nagai *et al.*, 2002; Pihlajamaki *et al.*, 2002).

The L4 proteins of isolates with either a *mefA* or *ermB* gene, which were all erythromycin resistant and some were telithromycin resistant, had no changes from the macrolide and ketolide sensitive TIGR 4 strain. None of the mutants except J III 8 had changes in the L22 protein. Therefore, in these cases the increase in telithromycin MICs were not due to alterations in the L4 or L22 proteins.

The J III 8 strain had the highest telithromycin MIC of >32mg/L and was *ermB* positive. The L22 amino acid mutation was from a lysine to a glutamine at amino acid 94. This change was located between two previously documented mutations in *S. pneumoniae*: G95D and A93E. The G95D mutation resulted in a 16 or 32-fold increase in telithromycin MIC and the A93E mutation, in combination with P91S and G83E mutations or a 23S rRNA mutation, enabled a 32-fold or 16-fold telithromycin MIC increase respectively (Canu *et al.*, 2002). Interestingly, the G95D mutation was also identified in combination with a 23S rRNA mutation in three clinical isolates from Japan (Farrell *et al.*, 2002). However, none of these changes caused telithromycin resistance.

The G95D and A93E amino acid changes are both from neutral amino acids to negatively charged amino acids. The K94Q change in J III 8 is from a positively charged amino acid to neutral. The positively charged residues of L22 primarily interact with the negatively charged phosphate group of the RNA (Unge *et al.*, 1998).

The mutation and the change in amino acid charge could affect the conformation and binding of the L22 to the 23S rRNA and its location in relation to the nascent peptide chain exit tunnel. This could in turn prevent telithromycin binding to the 23S rRNA. It is also interesting to note that the only glutamine present in the L22 protein was the one caused by this mutation. As yet it is unknown exactly how the K94Q mutation affects the L22 protein but it is certain that the mutation was present in a highly telithromycin resistant strain which had an *ermB* gene. Why the other strains with equally high telithromycin MICs did not have such a mutation is still however, unanswered, as too is the mechanism of telithromycin resistance in these strains.

As the NCTC 13593 strain could not be transformed with the mutated L22 gene, it is not possible to identify the importance of this mutation alone in the development of telithromycin resistance. Transformation depends on achieving a specialised cellular state know as competence. In *S. pneumoniae* this state is transitory and the development of competence under laboratory condition is influenced by many factors including culture pH, medium composition, the bacterial strain used and variations in temperature (Chen & Morrison, 1987). The lack of transformation could be attributed to the fact that the cells are not competent and therefore could not be transformed with the DNA.

The results of this study indicate that the L22 mutation occurred only at high-level telithromycin resistance. The lack of mutation in the other strains suggests that another mechanism or mechanisms of resistance must be at work. This mechanism is as yet unknown.

Chapter 9

Investigation of the *ermB* upstream region and induction of the *ermB* gene

9.1 Introduction

The upstream region of the *ermB* gene in *S. pneumoniae* consists of a promoter region, ribosome-binding site 1, a 27 amino acid control peptide and ribosome-binding site 2. The total region comprises 269 base pairs. In streptococci expression of the *ermB* gene may be inducible or constitutive. In staphylococci, inducible resistance is conferred mainly by 14- and 15-membered-ring macrolides but not lincosamides or 16-membered-ring macrolides or ketolides (Leclercq & Courvalin, 1991; Weisblum, 1995a; Bonnefoy *et al.*, 1997), recently resistance has also been induced by lincosamides and 16-membered-ring-macrolides (Clarebout *et al.*, 2001). Streptococci have cross-resistance between MLS_B antibiotics, which are all efficient inducers (Horinouchi *et al.*, 1983).

Mutations and deletions in the *ermB* upstream region have been associated with macrolide resistance changing from inducible resistance to constitutive resistance. The upstream region controls the methylase production and therefore this region determines how the gene is expressed, which in turn determines if the methylase gene may be turned on or off or just turned on in the case of constitutive resistance.

The upstream region of the *ermB* genes of the parent strain 02J1095 and mutant strains were investigated for alterations. Also, the ability of erythromycin and clarithromycin to induce telithromycin resistance in these strains was explored.

9.2 Materials and Methods

9.2.1 Bacterial strains

The *ermB* upstream regions of the parent strain 02J1095 and mutants J II 1, J III 1, J III 4, J III 5, J III 6, J III 7, J III 8 and J III 9, described in tables 8 and 13, were investigated. The strains 02J1095, J II 1, J III 1, J III 8 and the control strain *S. pneumoniae* NCTC 13593 were tested in the induction experiments.

9.2.2 PCR

The *ermB* upstream region was amplified using PCR. DNA was extracted from each strain as previously described. The forward primer was designed using the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi- bin/primer/primer3_www.cgi and the reverse primer was the same as the reverse primer used to amplify the *ermB* gene as described in table 24.

Table 24. *ermB* upstream primers

Primer	5′ to 3′	Product size
ermB upstream forward	GAA GCA AAC TTA AGA GTG TGT TGA	951 bp
ermB upstream reverse	AGT AA(CT) GGT ACT TAA ATT GTT TAC	

9.2.3 PCR reagents and cycle parameters

The PCR mixture was the same as that of the *ermB* gene PCR except the $MgCl_2$ concentration was 3mM and the forward primer *ermB* upstream forward was 46 pmol/μL. The cycle parameters were the same as those of the *ermB* PCR.

9.2.4 Agarose gel electrophoresis

The PCR products were run on 2% agarose gels stained and visualised as previously described.

9.2.5 Purification and Quantification of PCR Products

The *ermB* upstream region PCR products were purified as previously described using a QIAquick PCR purification kit.

9.2.6 Automated DNA Sequencing

The purified genes were sequenced by DNASHEF technologies, Edinburgh. The nucleotide sequences were compared to previously published sequences of the *S. pneumoniae ermB* gene using the BLAST online search facility (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The gene comparator was accession number X52632.

9.2.7 Transformation

To access the role of the mutated *ermB* upstream region in telithromycin resistance the resistant alleles of the *ermB* attenuator of J III 8 were amplified and purified. *Streptococcus pneumoniae* NCTC 13593 was used as the recipient strain. The competent cells preparation and transformation experiments were adapted from the method used by Canu *et al.*, 2002.

Competent cells were prepared as follows: *Streptococcus pneumoniae* NCTC 13593 was inoculated into 10mL of CAT broth (1000mL sterile distilled water, 5g NaCl (Sigma), 1g yeast extract (Sigma-Aldrich, UK), 5g Todd Hewitt broth (Oxoid), 10g enzymatic casein hydrolysate (Oxoid)) with 0.2% glucose (Sigma-Aldrich, UK) and 15mM K_2HPO_4 (BDH laboratories) (CAT I) and incubated in 5% CO_2 overnight.

One hundred micoliters of the overnight culture was inoculated into CAT I supplemented with 0.2% bovine serum albumin (Promega) and 1mM $CaCl_2$ (Sigma-Aldrich, UK) (CAT II). The broth culture was incubated in 5% CO_2 overnight. The cells were centrifuged at 3000g for 10 minutes in a Sorvall RT7 Plus centrifuge. The pellet was resuspended in 1mL of CAT II with 15% glycerol. The competent cells were split into 100µL aliquots and stored at -70°C.

For transformation the cells were thawed on ice and 10µL was added to 10mL of CAT II, which had been adjusted to pH 7.8. The mixture was then incubated in 5% CO_2 at 37°C for 15 minutes. Ten microliters of purified *ermB* upstream PCR product was added to 1mL of the culture and the reaction mixture was incubated in 5% CO_2 at 37°C for varying times of 1 hour 10 minutes, 3 hours and 18 hours. A negative control with 10µL of sterile milliQ water instead of purified PCR product was included in each run.

One hundred microliters of the resulting cultures were spread on Columbia agar plates supplemented with 5% defibrinated horse blood and telithromycin concentrations of 0.5mg/L or 1mg/L. Control plates with no antibiotic were also included in each assay. All plates were incubated in 5% CO_2 for 48 hours.

9.2.8 Induction experiments

i Disk Diffusion

The strains were each inoculated into 4.5mL of sterile distilled water and adjusted to a turbidity of 0.5 McFarland standard. Using a sterile swab each strain was spread onto separate Columbia blood agar plates. Disks containing 15μg of each of the following agents - erythromycin, clarithromycin and telithromycin - were placed onto the plate approximately 2cm apart. Each strain was tested in duplicate and the NCTC 13593 strain was used as a control.

ii Induction assays

In order to investigate inducibility further two induction assays were performed.

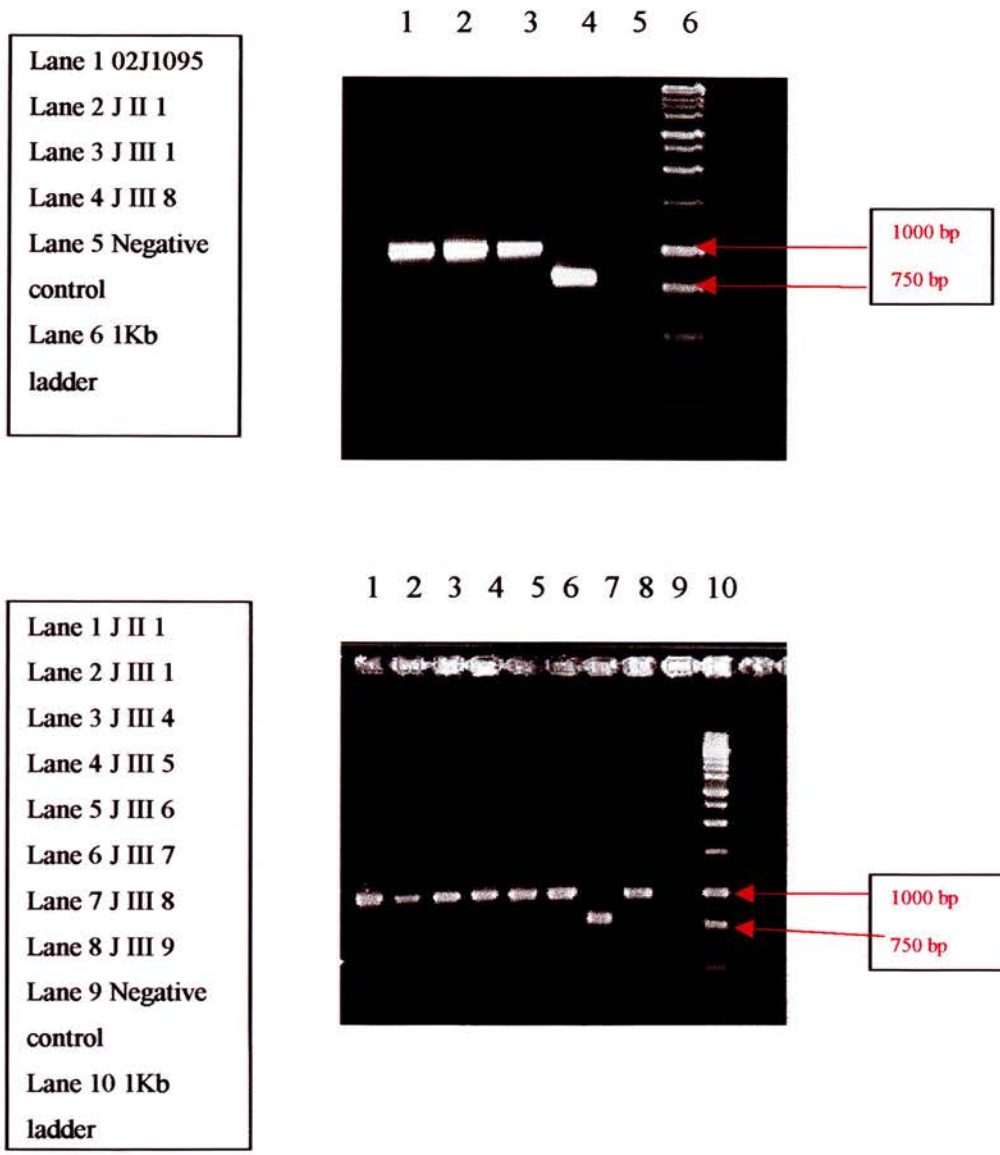
1. The parent 02J1095 was subcultured on erythromycin, clindamycin and lincomycin and the resulting mutants were tested for induction of telithromycin resistance. 02J1095 was inoculated into sterile saline and adjusted to 0.5 McFarland standard. One hundred microliters of the solution was spread on Columbia blood agar plates containing 0.5, 1 and 2 mg/L each of erythromycin, clindamycin, lincosamide and a control plate with no antibiotic and incubated in 5% CO_2 overnight. Colonies were randomly chosen from each plate and subcultured on plates with 2 mg/L of each antimicrobial agent and incubated overnight in 5% CO_2. The

MIC of telithromycin against these mutants was investigated as previously described. *Streptococcus pneumoniae* NCTC 13593 and *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 were all used as controls in the MIC tests.

2. The MICs of telithromycin in the presence of erythromycin (0.06 mg/l) of 02J1095, J II 1, J III 1 and J III 8 were determined as previously described. Control strains consisted of the *S. pneumoniae* NCTC 13593 and *S. aurues* NCTC 6571.

9.3 Results

Figure 24. *ermB* promoter region PCR gels



The corresponding bands of approximately 1000 base pairs of the *ermB* upstream region PCR products of 02J1095, J II 1, J III 1, J III 4, J III 5, J III 6, J III 7 and J III 9 were visualised on the gels as shown in figure 24. As illustrated in the gel photos in figure 24 the J III 8 band was just above the 750 bp band of the 1Kb ladder.

The sequencing results showed some anomalies not only in J III 8 but also in each of the other strains, in comparison to the *ermB* upstream sequence of the Tn*1545* of *S. pneumoniae* in the NCBI website. These are listed in table 25.

Table 25. Nucleotide mutations in the *ermB* upstream region.

Mutant strains (Telithromycin MIC mg/L)	Nucleotide change
02J1095 (0.06)	a292g c297t ta318, 319ag
J II 1 (1)	a292g c297t ta318, 319ag
J III 1 (4)	a292g c297t ta318, 319ag c324a
J III 4 (16)	a292g c297t ta318, 319ag c307t
J III 5 (32)	a292g c297t ta318, 319ag
J III 6 (32)	a292g c297t ta318, 319ag
J III 7 (>32)	a292g c297t ta318, 319ag g302a
J III 8 (>32)	207 bp deletion from 113 to 319
J III 9 (>32)	a292g c297t ta318, 319ag t322g

The 292g, 297t and 319g are all present in the *ermB* carrying plasmid pAM 77 of *Streptococcus sanguis* and the 318a mutation has previously been identified in an oral streptococcus. The nucleotide sequences of the ermB upstream regions 02J1095, J III 8, pAM77, pAMß-1, Tn*917* and Tn*1545* are compared in appendix 3. The c297t, a292g and the ta318, 319ag mutations have been located in clinical isolates of *S. pneumoniae*, which had inducible expression of the *ermB* gene. The mutations, which were present only in J III 1, J III 4, J III 7 and J III 9 have not been previously identified.

Deletions of large fragments of nucleotides upstream of the *ermB* gene similar to that found in J III 8 have been located in *S. agalactiae*, and *S. pneumoniae*, which had constitutive *ermB* expression. The plasmid pAMβ1 of *S. pneumoniae* also contained the same deletion as these strains (Rosato *et al.*, 1998; Rosato *et al.*, 1999). However, in these strains this deletion removed the first ribosome binding site (rbs) and the control peptide but J III 8 had a deletion of the second rbs and the control peptide.

In the transformation experiments growth was observed at each time point on the non-selective plates with no telithromycin. However no colonies were present on the transformant selective plate containing telithromycin from any of the time points. Therefore the effects of the *ermB* attenuator region mutation alone on the development of telithromycin resistance could not be determined as the mutated *ermB* attenuator region was not transformed into the telithromycin sensitive NCTC 13593 strain.

9.3.1 Induction experiment results

Figure 25. Parent strain 02J1095 with discs of erythromycin, clarithromycin and telithromycin.

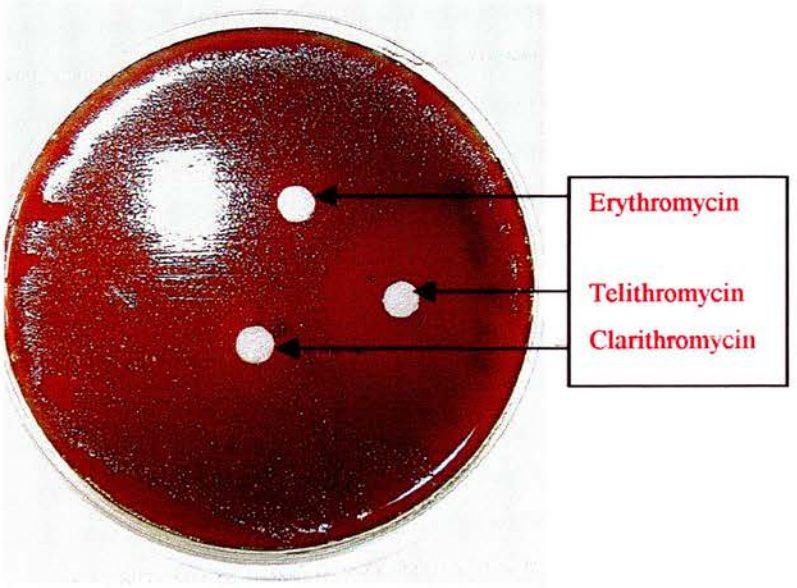
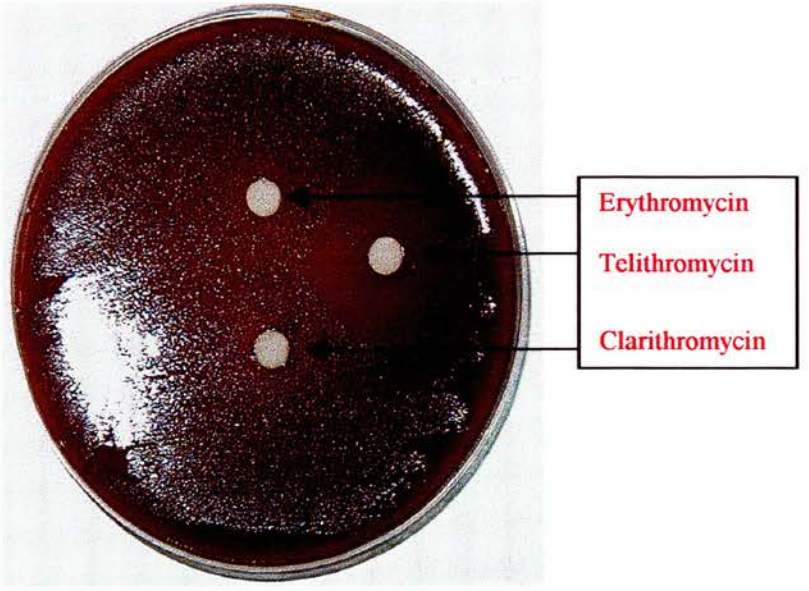


Figure 26. Mutant J II 1 with discs of erythromycin, clarithromycin and telithromycin.



On both plates, with 02J1095 and J II 1, the zone of inhibition around telithromycin had two straight edges, one each from erythromycin and clarithromycin, which can be seen in figures 17 and 18. The D-shaped zone indicates that the antimicrobial agent to the left of the D induces resistance. From these photographs it can be seen that erythromycin and clarithromycin both induce telithromycin resistance. The growth of J III 1 to J III 9 were either up to the discs or within 1mm of the discs and so were recorded as resistant to the 3 antimicrobial agents and as such had constitutive expression of the *ermB* gene.

9.3.2 Induction assays results

The MICs of the mutant strains derived from growth of 02J1095 on erythromycin, clindamycin or lincomycin are shown in table 26. (E2, L2 and C2 are the mutant strains subcultured on 2mg/L of erythromycin, lincomycin or clindamycin respectively. No antibiotic is the negative control, which was subcultured on blood plates without antibiotic). The telithromycin MICs of the strains tested in the presence of 0.06mg/L of erythromycin are shown in table 27.

Table 26. Telithromycin MIC results of strains mutated in the presence of erythromycin, lincomycin and clindamycin.

Strain	Telithromycin MIC (mg/L)
02J1095	0.06
No antibiotic	0.12
E2 1 to 3	0.12
L2 1 to 3	0.12
C2 1 to 3	0.12

The set of MICs shown in table 26, indicate that when 02J1095 was subcultured in the presence of erythromycin, lincomycin or clindamycin, the telithromycin MIC did not increase any more for the "mutant" strains than for the negative control strain, which had been subcultured on antibiotic-free plates. They did however, all increase by one doubling-dilution but this is not as a result of changes induced by the antimicrobial agents but merely an inaccuracy of the way in which MICs are tested.

Table 27. Telithromycin MIC results of 02J1095 mutants in the presence of erythromycin.

Strain (Telithromycin MIC mg/L)	Telithromycin MIC (mg/L) with 0.06mg/L of erythromycin per plate
02J1095 (0.06)	0.25
J II 1 (1)	1
J III 1 (4)	32
J III 8 (>32)	>32

With regard to the second MIC test, the MICs of 02J1095 and J III 1 did increase as shown in table 27. The telithromycin MIC of the parent 02J1095 increased by 2

doubling dilutions, from 0.06mg/L to 0.25mg/L and that of J III 1 increased by 3 doubling dilution from 4mg/L to 32mg/L. While the MICs of these 2 strains did increase the telithromycin MIC of J II 1 remained the same. Therefore, although the disc diffusion results suggest that erythromycin induces telithromycin resistance, this conclusion has not been backed up conclusively by the MIC assays.

9.4 Discussion

Deletions in the *erm* gene attenuator region in *S. pyogenes*, *S. pneumoniae* and *S. agalactiae* have all been associated with constitutive expression of their Erm methylases. In *S. pyogenes* deletions of 163 base pairs or 6 base pairs and a duplication of 101 base pairs in the *erm(TR)* upstream region resulted an increase in clindamycin MIC from 1mg/L to 128mg/L when transformed into *E. coli*. (Fines *et al.*, 2001). The *S. pyogenes* strains with mutated attenuators were mutants which had been selected on clindamycin, the parent strain was inducibly intermediate to erythromycin and fully susceptible to clindamycin. Tait-Kamradt *et al.*, (2001) described two *S. pneumoniae* clinical isolates with truncated *ermB* leader peptides of 15 and 19 amino acids. These 2 strains had telithromycin MICs of 1mg/L and 256mg/L respectively and both also had 3 amino acid changes in the gene itself. The strain with the 256mg/L telithromycin MIC also contained an L4 riboprotein amino acid mutation of $_{69}$GTG$_{71}$ to $_{69}$TPS$_{71}$.

The results of the PCR experiments of the *ermB* upstream region performed with the parent 02J1095 and the mutants derived from it showed that the J III 8 strain had a

large deletion of 206 bps in this section removing the second ribosome binding site and the control peptide. This result is very similar to that found with the clindamycin resistant *S. pyogenes*. Therefore, it is possible that a mechanism of resistance exists that alters the *ermB* attenuator of strains inducibly resistant to erythromycin in order to confer resistance to other antimicrobial agents within the same group, such as clindamycin and telithromycin. The results of Tait-Kamradt *et al.*, (2001) are also similar to the findings of this study in that the telithromycin resistant strains both had truncated regions upstream from the *ermB* gene. The other mutants investigated in this study, which were also highly telithromycin resistant did not however have such a deletion. Therefore, while the mutation in the *ermB* attenuator may be at least partly responsible for telithromycin resistance in J III 8, this is not the case for the other 5 highly telithromycin resistant strains.

The mutated nucleotides at positions 292, 297 and 319 of the strains investigated are the same nucleotides as those of plasmid pAM 77 from *S. sanguis* and the nucleotide change at position 318 was found previously in an oral streptococci. These mutations were in the parent 02J1095 and all the mutants, except J III 8, and as such do not appear to be involved in the development of telithromycin resistance. It is, however, interesting to note the nucleotide changes at 318 and 319 are just at the end of the deletion in J III 8. Two previously reported *S. pneumoniae* isolates with the same 318 and 319 mutations were both inducibly erythromycin resistant but sensitive to the ketolide HMR 3004 (Rosato *et al.*, 1998). The other single mutations in J III 1,

J III 4, J III 7 and J III 9 have not previously been associated with changes in resistance patterns. But as each strain has a different mutation it is not likely that these mutations individually lead to telithromycin resistance.

In order to investigate the effect of the truncated *ermB* attenuator region on telithromycin resistance the corresponding PCR product would need to be transformed into a sensitive strain and the telithromycin MIC of the transformant tested. As the transformation experiments did not work it is impossible to assess the effect of the individual mutation on telithromycin resistance. The lack of transformation is probably due to problems with the competency of the telithromycin sensitive NCTC 13593 strain rather than with the mutated *ermB* attenuator region DNA. The concentration at which *S. pneumoniae* develops competence varies with the composition of the medium, the bacterial strain used and the medium pH (Chen & Morrison, 1987). Any one of these factors could have contributed to the prevention of transformation of the NCTC 13593 strain with the mutated *ermB* attenuator region DNA.

The disk diffusion experiments indicated from the D-shaped zones of inhibition around the telithromycin disc that erythromycin and clarithromycin are both inducers of telithromycin resistance in the strains 02J1095 and J II 1. However, when the 02J1095 strain was subcultured in the presence of erythromycin the mutants did not result in an increase in telithromycin MIC in comparison to the negative control. When the telithromycin MICs of the four strains were investigated in the presence of erythromycin the telithromycin MICs of 02J1095 and J III 1 did increase but that of

J II 1 did not. Thus there is a conflict between the 3 sets of results as to the extent that erythromycin induces telithromycin resistance. It is possible that the differences arise due to the varying concentrations of erythromycin used in each experiment, but this is unlikely. There does however, appear to be a relationship between erythromycin and telithromycin resistance but further studies are required to investigate if this is the case with a larger number of isolates.

When the results of the experiments are taken together the overall conclusion is that erythromycin and clarithromycin induce telithromycin resistance but that this cannot be verified by the MIC assays. Also, the high-level telithromycin MICs of the mutants are not all caused by the same mutation in the *ermB* attenuator. Although the large deletion in J III 8 is such that it is probably part of the mechanism used by this strain to overcome telithromycin. The mechanism(s) of resistance in the other mutants, however, remain to be identified.

Chapter 10

Investigation of the *mef* upstream and downstream nucleotide regions

10.1 Introduction

The chromosomal insertion elements containing the *mef* gene in *S. pneumoniae* have recently been characterised. The region downstream from *mef* is an open reading frame (orf) called *mel*. The predicted protein of this orf showed 36.2% amino acid identity to the erythromycin resistance ATP-binding protein MsrA of *Staphylococcus epidermidis*. Reverse-transcriptase (RT)-PCR demonstrated that *mefE* and *mel* were co-transcribed (Gay & Stephens, 2001). Tait-Kamradt has also discovered that knocking out the *mef* gene from the *mega* operon stops expression of both *mef* and *mel* whereas knocking out the *mel* gene also eliminates macrolide resistance but not transcription of *mef* (personal communication). The region upstream from the *mef* gene contained a 944-bp region with no predicted orf. It did, however, contain a putative promoter region. Deletions or mutations within the promoter region, control peptide region and ribosome binding site upstream from *ermB* have been associated with changes in gene expression from inducible to consititutive. Therefore, both the upstream region containing the promoter and the downstream *mel* region are

necessary for *mef* function. Changes in the *mef* encoded efflux pump could be controlled by alterations of either of these two sections.

The aim was to investigate if the telithromycin resistant *mef* positive mutants contained the same *mel* and upstream regions as the sensitive strains.

10.2 Materials and Methods

10.2.1 Bacterial strains

The parent 02J1175 and the mutants M I 3, M II 15, M III 3 and M IV were analysed, described in tables 9 and 13.

10.2.2 PCR

The *mel* gene and *mef* upstream region of the bacterial strains were amplified using PCR. DNA was isolated from each strain as described in the previous chapters. The primers used to amplify the *mel* genes and upstream regions were previously described by Gay and Stephens (2001) and Sutcliffe *et al.*, (1996b), except for the *mef* upstream forward primer, shown in table 28. This was designed using the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi . The *mef* upstream reverse primer was the same as the reverse primer used to amplify the *mef* gene and

the *mel* forward primer was the forward primer used to amplify the *mef* gene as described in table 28. The *mef* upstream region PCR product was 614 bps and the *mel* PCR product was 1680 bps.

Table 28. *mega* operon primers.

Primer Name	Sequence 5′ to 3′
mef upstream forward	GAG CAT TCA TTA GTT ACG GTG AGG
mef upstream reverse	TTC TTC TGG TAC TAA AAG TGG
mel forward	AGT ATC ATT AAT CAC TAG TGC
mel reverse	CTT CAC GGT CTA AAT GGC TCG

10.2.3 PCR reagents

The PCR reaction mixtures consisted of the same contents as those for the *mefA* gene PCR mixture except for the $MgCl_2$ concentration of the *mel* PCR mixture, which was 1.5 mM and the primers. The *mef* upstream forward primer was 60 pmol/μL and the *mel* reverse primer was 67 pmol/μL.

10.2.4 PCR cycle parameters

The reaction mixture was set up in a Techne Thermal Cycler under the same parameters as the *mefA* amplification for the *mef* upstream region PCR and the conditions described in table 29 for the *mel* PCR.

Table 29. Cycle parameters for the *mel* PCR amplification

Number of cycles	Temperature (°C)	Time (minutes)
1	95	3
35	95	0.5
	60	0.5
	72	1
1	72	5

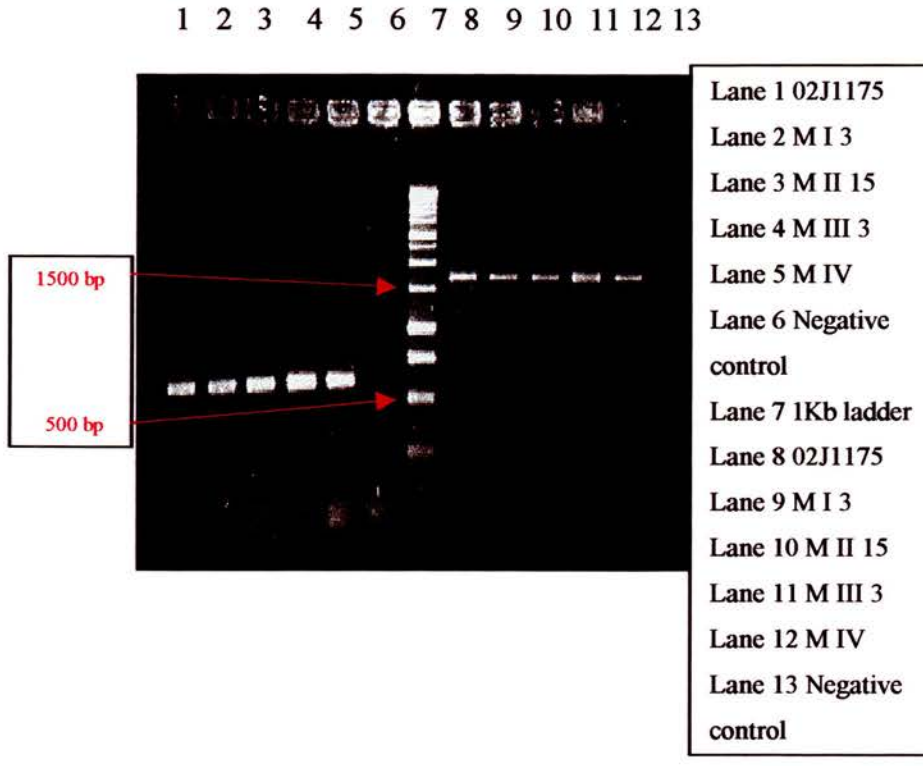
10.2.5 Agarose gel electrophoresis

The PCR products were run on 2% agarose gels, stained and visualised as previously described.

10.3 Results

The *mef* upstream region PCR products and the *mel* genes of the strains were amplified and the corresponding PCR bands at 614 bp and 1680 bp respectively were visualised on the agarose gel as shown in figure 27. Lanes 1 to 6 are the PCR products of the *mef* upstream region and lanes 8 to 13 are the *mel* PCR products.

Figure 27. *mef* upstream region PCR products and *mel* PCR products gel.



There were no changes in the sizes of the *mef* upstream region, containing the promoter, or the downstream *mel* region in any of the mutants.

10.4 Discussion

The sizes of the upstream and downstream regions from the *mef* genes suggest that no large nucleotide deletions have occurred. However, nucleotide changes could be present but these would not have been detected by PCR amplification. Likewise, the PCR gels would not have shown deletions of small numbers of nucleotides. The presence of the *mel* genes in these strains confirms previous studies of the *mef* operon, which suggested the presence of the *mel* gene downstream from the *mef*

gene. As the primers were not specific for either the *mega* element or the transposon Tn*1207.1*, which also contains the *mef* gene, it is still unclear as to which element is present. From this study it can be seen that deletions of large numbers of nucleotides from either the upstream or downstream nucleotide regions of the *mef* gene are not responsible for the increase in telithromycin MIC and resistance. Further studies, such as sequencing of the two regions either side of the *mef* gene are required to determine if nucleotide mutations or deletions of small numbers of nucleotides are present. A change in the efflux pump protein encoded by the *mef* gene and/or the transporter mediated by *mel* could be required to change the efflux pump sufficiently to also efflux telithromycin.

Chapter 11

Discussion

Telithromycin has been reported as the antimicrobial agent that will overcome MLS_B resistance and be used against respiratory tract infections with great activity. The novel features of telithromycin enable it to overcome the macrolide resistance mechanisms used by *S. pneumoniae*. Furthermore, as telithromycin does not induce macrolide resistance its use will not increase the amount of macrolide resistance already present, particularly in *S. pneumoniae* (Bonnefoy *et al.*, 1997). Macrolide resistance is currently a problem in many countries and the prospect of an antimicrobial agent capable of combating macrolide resistance is of great hope, particularly in countries with high levels of macrolide resistance and also in countries such as the UK where macrolide resistance is increasing.

The aims of this study were firstly, to investigate the efficacy of telithromycin against *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*, secondly, to investigate the way in which telithromycin works by generating telithromycin resistant mutants and thirdly to identify the mechanism(s) of resistance generated. As telithromycin has already been launched in some countries, it is vital to know how easily resistance to telithromycin may be generated, not only from the macrolide sensitive *S. pneumoniae* populations, but also the main target of telithromycin; the macrolide resistant *S. pneumoniae*.

In order to access the efficacy of telithromycin it was tested *in vitro* in comparison to a range of antimicrobial agents against strains of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. The activity of telithromycin was also tested specifically against macrolide resistant *S. pneumoniae* from around the world with different resistance mechanisms. Telithromycin had high activity against both the clinical isolates of *S. pneumoniae* gathered from various UK centres and the macrolide resistant *S. pneumoniae* strains. Twenty per cent of the UK isolates and 100% of the macrolide resistant isolates were erythromycin resistant, but there was no resistance to telithromycin in either group of *S. pneumoniae*. From these results it appeared that telithromycin would be an excellent choice of antimicrobial agent to combat macrolide resistant *S. pneumoniae*. The erythromycin resistance breakpoint in *S. pneumoniae* is ≥1mg/L (MacGowan & Wise 2001) and that of telithromycin ≥4 mg/L (NCCLS, 2000). Even if both telithromycin and erythromycin resistant strains were required to have an MIC of ≥1 mg/L then there would only be 1% or 1 strain in the UK isolates which would be telithromycin resistant and approximately 10% (9.6%) of the macrolide resistant strains, which would be telithromycin resistant. Therefore, the strains that are already macrolide resistant have higher telithromycin MICs than those that are sensitive and so appear to be able to prevent telithromycin inhibiting *S. pneumoniae* to it's full extent.

Three *S. pneumoniae* strains were used in the mutation studies. Two were macrolide resistant and contained either an *ermB* gene or a *mefE* gene; the third was macrolide sensitive and was negative for both genes. The selection of telithromycin resistant strains varied not only by their telithromycin MICs but also the frequency of

isolation. The mutants derived from the NCTC 13593 (macrolide sensitive) parent were not telithromycin resistant but the MICs increased from 0.016mg/L for the parent to 0.5mg/L for the final generation mutants. This was the lowest telithromycin MIC of all the final generation mutants created. However, the NCTC 13593 mutants had the highest mutation frequencies, so although the MICs were not telithromycin resistant the mutants were the easiest to propagate and resulted in the largest number of isolates. Therefore, even though the macrolide sensitive parent strain did not produce telithromycin resistant mutants, it did produce mutants with elevated telithromycin MICs in large numbers.

The mutants with the highest telithromycin MICs were generated from the *ermB* positive parent. The second-generation mutants had telithromycin MICs from 4mg/L to >32mg/L and so were all telithromycin resistant. The second-generation mutants derived from the *mefE* positive parent had telithromycin MICs of 2mg/L or 4mg/L. These results indicated that it only required two generations for an *ermB* positive or *mefE* positive strain to become telithromycin resistant. Therefore, while telithromycin had excellent activity against both *ermB* positive and *mefE* positive *S. pneumoniae* resistance to telithromycin developed over two generations.

The mutation frequencies of the *ermB* positive, the *mefE* positive and the macrolide sensitive parents all differed. The first generation mutation frequency of the *ermB* positive mutants was 1×10^{-3},which then decreased to 1×10^{-6} for the next generation. This suggested that a two-fold mutation occurred, the first mutation in order to achieve the first generation mutants and a further mutation to obtain the

second-generation mutants with the higher telithromycin resistance. For the *mefE* positive and the macrolide sensitive mutants, the mutation frequencies are within 10-fold of each other for each generation and thus the strains adapted gradually to telithromycin.

To date telithromycin resistant mutants have usually been derived from macrolide susceptible parent strains, which could explain why the results of this study vary from those previously published. A macrolide susceptible strain required 24 passages on telithromycin for a mutant with a telithromycin MIC of 8mg/L to emerge, whereas a strain with an *ermB* gene required only 3 passages in telithromycin to result in a mutant with a telithromycin MIC of 8mg/L (Davies *et al.*, 2000b).

The mechanism(s) used by the *mefE* positive strains to become resistant is not stable as the mutants reverted to the lower telithromycin MICs when telithromycin selective pressure was removed. This has previously been noted by Davies *et al* (2000b), when the macrolide and telithromycin MICs of *mef* positive *S. pneumoniae* mutants generated by passage on MLS_B antibiotics and telithromycin reverted back to baseline MICs after 10 passages on antibiotic-free media. The resistance mechanism used by the *mefE* positive strains generated from 02J1175 appeared to be induced by telithromycin as the revertant telithromycin MICs returned to those of the original mutants when they were reintroduced to a telithromycin environment. The *ermB* positive mutants were stable; their resistance mechanism(s) were stable and were not turned off by removing the selective pressure of telithromycin. This has also

previously been found in macrolide and telithromycin resistant *S. pneumoniae* by Davies *et al* (2000b).

When the mutants were checked for *ermB* or *mefE* genes, the macrolide sensitive mutants contained neither gene, the mutants derived from the *ermB* positive parent contained only the *ermB* gene and those generated from the *mefE* positive parent only the *mefE* gene. Therefore, cross-contamination or transfer of either gene did not occur. The nucleotide sequences of the selected *ermB* positive mutants revealed no changes from the parent strain. The changes that were identified did not appear to have a role in telithromycin resistance as they were in telithromycin sensitive and resistant strains.

The disc diffusion induction experiments showed that telithromycin resistance could be induced by erythromycin or clarithromycin for the *ermB* positive parent, 02J1095 and mutant J II 1. The resistance levels of the *ermB* positive mutants with higher telithromycin MICs strains were constitutively expressed, as they had no zones of inhibition around the three discs. This indicated that they were resistant to all three antimicrobial agents. The results of the different induction experiments varied but disc diffusion is used as the primary experiment to test for induction of MLS_B resistance. If the disc diffusion results are taken alone then erythromycin and clarithromycin induce telithromycin resistance. The clinical implications of this finding are that telithromycin resistant strains could emerge from *ermB* positive strains that had been induced by either erythromycin or clarithromycin. Therefore, the main accolade of telithromycin, its ability to inhibit macrolide resistant

S. pneumoniae that were also clindamycin resistant, would be removed.

Deletions in the *erm* gene attenuator region in *S. pyogenes*, *S. pneumoniae* and *S. agalactiae* have all been associated with constitutive expression of their Erm methylases. A number of nucleotide changes were present in the region upstream from the *ermB* gene in all *ermB* positive strains derived from 02J1095, which are shown in appendix 3. In the *ermB* positive parent and the mutants (except J III 8) four nucleotide changes were present, which were the same in each strain. Two of these mutations were at nucleotides 318 and 319. The second-generation J III 8 mutant had a 207 base pair deletion in the *ermB* upstream region, which removed the control peptide and the second ribosome-binding site. The cut was before nucleotide 113 and after nucleotide 319. It appeared that the telithromycin sensitive *ermB* positive, parent strain may have characteristic mutations, which enabled it to develop telithromycin resistance by having altered nucleotides and providing a nucleotide sequence that could be cut and lead to a truncated *ermB* upstream region. When the 318, 319 nucleotides were mutated to AG and the sequence is read from 5' to 3' both the four nucleotides just before the cut at 113 and before the cut at 319 would read GATT. This implies that the mutated G is required as a signal to cut at that position.

The mutated nucleotides of the strains investigated at positions 292, 297 and 319 are the same nucleotides as those of plasmid pAM 77 from *S. sanguis* and the nucleotide change at position 318 was found previously in an oral streptococci (Rosato *et al.*, 1998; Rosato *et al.*, 1999) as shown in appendix 3. These mutations were in the parent 02J1095 and all the mutants, except J III 8, and as such do not appear to be

involved in the development of telithromycin resistance. It is interesting to note the nucleotide changes at 318 and 319 are just at the end of the deletion in J III 8. Two previously reported *S. pneumoniae* isolates with the same 318 and 319 mutations were both inducibly erythromycin resistant but sensitive to the ketolide HMR 3004 (Rosato *et al.*, 1998). The nucleotide in the *ermB* upstream region at nucleotide positions 292, 297, 318 and 319 were the same as those of *S. sanguis* pAM77 *ermB* upstream region. Thus, it appeared that this *ermB* upstream region had either adapted in the same way as that of *S. sanguis* pAM77 *ermB* upstream region or at some time the *ermB* plasmid of *S. sanguis* was transferred to the *ermB* positive parent 02J1095. These mutations are not present in the upstream region of the *ermB* gene carried on Tn*1545*.

The deletion in J III 8 removed the control peptide and one of the ribosome binding sites. This therefore, ensures that the mRNA read from the DNA would not produce the control peptide. The ribosome-binding site for transcription of the *ermB* gene would also differ and thus change the ErmB methylase production. This deletion may be partly responsible for the high telithromycin resistance of J III 8. However, this type of deletion cannot be responsible for all telithromycin resistance in *S. pneumoniae* as it was not present in the other highly telithromycin resistant mutants. Thus, more than one mechanism of telithromycin resistance exists even within a group of strains derived from the same parent.

The J III 8 strain deletion of 207 bps was very similar to that found with clindamycin resistant *S. pyogenes*. In *S. pyogenes* deletions of 163 base pairs or 6 base pairs and a

duplication of 101 base pairs in the *erm(TR)* upstream region resulted an increase in clindamycin MIC from 1mg/L to 128mg/L when transformed into *E. coli*. (Fines *et al*., 2001) The *S. pyogenes* strains with mutated attenuators were mutants, which had been selected on clindamycin, the parent strain was inducibly intermediate to erythromycin and fully susceptible to clindamycin. Therefore, it was possible that a mechanism of resistance exists that alters the *ermB* attenuator of strains inducibly resistant to erythromycin in order to confer resistance to other antimicrobial agents within the same group, such as clindamycin and telithromycin.

Tait-Kamradt *et al*., (2001) described two *S. pneumoniae* clinical isolates with truncated *ermB* leader peptides of 15 and 19 amino acids. These 2 strains had telithromycin MICs of 1mg/L and 256mg/L respectively and both also had 3 amino acid changes in the gene itself. The strain with the 256mg/L telithromycin MIC also contained an L4 riboprotein amino acid mutation of Glycine, Threonine and Glycine at amino acids 69 to 71 to Threonine, Proline and Serine respectively. The results of Tait-Kamradt *et al*., (2001) are also similar to the findings of this study in that the telithromycin resistant strains both had truncated regions upstream from the *ermB* gene. The other 02J1095 mutants investigated in my study, which were also highly telithromycin resistant, did not however have such a deletion. Therefore, while the mutation in the *ermB* attenuator may be at least partly responsible for telithromycin resistance in J III 8, this is not the case for the other 5 highly telithromycin resistant strains. The other single mutations in J III 1, J III 4, J III 7 and J III 9 have not previously been associated with changes in resistance patterns, but as each strain has

a different mutation it is not likely that these mutations individually lead to telithromycin resistance.

In order to investigate the effect of the truncated *ermB* attenuator region on telithromycin resistance, the corresponding PCR product would need to be transformed into a sensitive strain and the telithromycin MIC of the transformants tested. As the transformation experiments did not work it is impossible to assess the effect of the mutation alone on telithromycin resistance.

Due to the discovery of this large deletion in the *ermB* gene upstream region in J III 8, the *mefE* upstream and downstream regions were also amplified. No deletions large enough to be noticed on the PCR product agarose gel were detected in either region but sequencing of these regions would be required to determine if small nucleotide deletions or mutations were present. The *mefE* upstream region contains a putative promoter and the downstream region an orf called *mel*, which is co-transcribed with *mefE* and could be the pump behind the *mefE* efflux pump as it has amino acid homology to the erythromycin ATP-binding protein MsrA of *S. epidermidis*. Therefore, alterations in either region would alter *mefE* expression and could change to also efflux telithromycin. As the *mef* positive mutants were not stable the mechanism(s) used by these strains could have such a large cost to the cell that it is only turned on as and when required. This idea would fit into the theory that extra pumping pressure or an alteration in pump size, or both was required by these strains to become telithromycin resistant.

Further regions of the telithromycin resistant mutants were probed for alterations that have previously been responsible for macrolide resistance and increased telithromycin MICs or resistance. The peptidyl transferase region of the ribosome, containing the domains II and V of the 23S rRNA and the L4 and L22 riboproteins are the main areas of interaction for the macrolides and ketolides, such as telithromycin. They are also the main regions of interest to date for macrolide resistance in *S. pneumoniae*. The area of consequence with regard to telithromycin in domain II is the hairpin 35. A deletion in one adenine in the series of four located at positions 749 to 752 resulted in a 500-fold increase in the telithromycin MIC of a *S. pneumoniae* strain. In this case it became resistant to telithromycin (4 mg/l) (Canu *et al.*, 2002). Previously a single point mutation (U754A) in a laboratory strain of *E. coli* resulted in the cells being resistant to telithromycin (Xiong *et al.*, 1999). For domain V there is less specificity with regard to one macrolide resistant region. Mutations have been located at nucleotides 2058, 2059, 2062 and 2611 (Tait-Kamradt *et al.*, 2000b; Depardieu & Courvalin, 2001; Farrell *et al.*, 2002; Pihlajamäki *et al.*, 2002). These ribosomal mutations were identified in macrolide resistant clinical isolates, which did not contain the *ermB* or *mefA* genes.

Streptococcus pneumoniae has four copies of the 23S rRNA genes (Tait-Kamradt *et al.*, 2000b). These were amplified and sequenced from the *ermB* and *mefE* mutants, in order to identify any alterations that may have prevented telithromycin binding to its site of interaction. Mutations in domain V have been associated with increased telithromycin MICs and alterations in domain II with telithromycin resistance (Canu *et al.*, 2002). No mutations were present in any of the mutants investigated in this

study. Therefore, these results call into question the importance of previous mutations associated with increased telithromycin MICs but not high-level telithromycin resistance in the development of telithromycin resistance. This implies that telithromycin resistance is mediated by alterations and adaptations in other regions of the cell. There is also the possibility that alterations in other regions of the 23S rRNA, which had a lower cost to the strains, may have prevented telithromycin from binding and so there was no need to alter the domains II or V of the 23S rRNA.

Riboproteins L4 and L22 both line the nascent peptide exit tunnel of the ribosome (Gabashvili *et al.*, 2001). They have been associated with the erythromycin and telithromycin binding sites of the ribosome. Mutations in both these riboproteins have been associated with decreased macrolide and ketolide activity (Sutcliffe *et al.*, 2000; Tait-Kamradt *et al.*, 2001; Canu *et al.*, 2002; Farrell *et al.*, 2002,). The L4 protein has been associated with large increases in telithromycin MIC in *S. pneumoniae*. An insertion of 6 amino acids into a highly conserved area of ribosomal L4 protein ($_{63}$KPWRQKGTGRAR$_{74}$) has been proven to cause a 500-fold increase in the MIC of telithromycin (Tait-Kamradt *et al.*, 2000b). Changes within the L22 protein amino acid sequence have also been reported as a cause of increased telithromycin MIC. A telithromycin MIC increase from 0.008mg/L to 0.25mg/L was associated with three simultaneous amino acid mutations: Alanine-93 to Glutamic acid-93, Proline-91 to Serine-91 and Glycine-83 to Glutamic acid-83 and a Glycine-95 to Aspartic acid-95 mutation was associated with telithromycin MIC increases from 0.004mg/L and 0.008mg/L to 0.12mg/L (Canu *et al.*, 2002).

The exit tunnel lined by L4 and L22 is thought to be blocked by erythromycin and therefore most probably telithromycin (Gabshvili *et al.*, 2001). Mutations in either riboprotein could prevent telithromycin from blocking this tunnel. In this study no mutations were identified in the L4 riboprotein. One mutation in the L22 was located in the *ermB* positive J III 8 mutant. This resulted in a lysine to glutamine amino acid change at position 94. Mutations at amino acids 93 and 95 have previously been associated with increased telithromycin MICs (Canu *et al.*, 2002).

The tip of the L22 is elongated and is mainly positively charged. The three amino acids contributing to this positive charge at the L22 tip in *Thermus thermophilus* are three arginines at amino acid positions 88, 90 and 92 (Unge *et al.*, 1998). These correspond to an arginine, a lysine and a serine respectively in *S. pneumoniae*. Both arginine and lysine are positively charged and serine is neutral. The positive tip of the L22 interacts with the negatively charged RNA. Therefore, alterations of amino acid size or charge could change the tip of the L22 such that it did not bind to the RNA or result in a conformation change of the L22 riboprotein; either of these outcomes could then prevent telithromycin binding to the 23S rRNA.

In J III 8 the amino acid change from lysine to glutamine resulted in a charge change from a positive lysine residue to a neutral glutamine amino acid residue. The lysine corresponds to the arginine at position 90 in *Thermus thermophilus*, one of the three amino acids responsible for the positively charged tip of L22. The tip of the L22 extends into the nascent peptide chain exit tunnel. A change in charge would result in

the L22 not binding to the RNA inside the tunnel, which could then block telithromycin binding.

From the results generated in this study it can been seen that the highly telithromycin resistant *ermB* positive mutant J III 8 developed at least two mechanisms of resistance to telithromycin. One was a change in the ErmB methylase control sequence and the other an alteration in the L22 riboprotein. If the *ermB* upstream mutation lead to either an increase in ErmB methylase production or a change in the position of methylation away from the adenine at nucleotide position 2058 in the domain V of the 23S rRNA to another position more closely associated with telithromycin binding, then this could block telithromycin binding. If this was the case then there would have been no need to alter the nucleotides in the domains II or V of the 23S rRNA and the L22 mutation would have been a secondary mutation as a reaction to the ErmB methylation rather than a mutation caused directly by the adaptation to telithromycin exposure.

The converse may also be true that the L22 mutation occurred as a telithromycin resistance mechanism and blocked not only telithromycin but also the ErmB methylase from binding to adenine 2058. Erythromycin resistance mutations in L22 riboprotein perturbed the conformation of residues in domains II, III and V (Gregory & Dahlberg, 1999). Thus, the *ermB* upstream region was altered in order to allow ErmB methylase to bind in a slightly different fashion or a larger amount produced as less methylation could occur due to the altered L22.

A third theory is that both the *ermB* upstream mutation and the L22 mutation are merely further adaptations of the main resistance mechanism. This new mechanism however, does not cause mutation in the 23S rRNA or the *ermB* gene itself. It would seem feasible as no *ermB* upstream mutation or L22 mutation were located in the other *ermB* positive telithromycin resistant mutants.

The *ermB* positive telithromycin resistant mutants, other than J III 8, did not have either the *ermB* upstream region deletion or the L22 amino acid change. Therefore, it appeared that these two mutations were linked together and were not two separate steps involved in telithromycin resistance development. No mutations were located in any of the mutants generated, in the 23S rRNA domains II and V, the L4 riboprotein or the *ermB* gene and only J III 8 contained mutations in the L22 riboprotein and the *ermB* upstream region. Therefore, none of the mechanisms previously associated with macrolide resistance in *S. pneumoniae* or the ribosome binding sites were adapted to provide these strains with a selective advantage over telithromycin. It appeared that a new mechanism of resistance has been developed or selected for to overcome telithromycin. This mechanism could be require a two–step mutations in *ermB* positive strains as the telithromycin MIC increased to 1mg/L for the first generation mutants and >32mg/L for the second generation with mutation frequencies decreasing from 10^{-3} to 10^{-6}.

The *mefE* positive mutants also did not have alterations in the 23S rRNA or the L4 or L22 riboproteins. In these strains it is most probable that the efflux pump mechanism adapted to efflux telithromycin, as the mutants telithromycin MICs increased steadily

over the generations. However, the mechanism(s) involved must have had a high cost to the cell as the mechanism(s) was turned off when the pressure of telithromycin was removed. No mutations in the *mef* gene or the *mef* chromosomal elements of *S. pneumoniae* have been associated with either macrolide or ketolide resistance to date.

11.1 Conclusions

Telithromycin had excellent *in vitro* activity against macrolide sensitive and resistant *Streptococcus pneumoniae*. Telithromycin resistant *ermB* positive and *mefE* positive mutants developed *in vitro* within two generations. When the strains were macrolide sensitive the telithromycin MIC also increased greatly (30-fold) but did not result in resistance. Erythromycin and clarithromycin induced telithromycin resistance. A deletion of 207 base pairs in the *ermB* upstream region and a lysine to glutamine amino acid residue mutation at position 94 in L22 were present in a highly telithromycin resistant *ermB* positive mutant. The final conclusion of this study is that telithromycin must be used with caution against MLS_B and macrolide resistant *S. pneumoniae*. The results generated in this study suggested that telithromycin resistant *S. pneumoniae* will develop, particularly in countries with macrolide resistant *S. pneumoniae*.

Appendices

Appendix 1

Streptococcus pneumoniae MICs

Strains	Telith CO_2	Telith air	Ery	Clar	Azith	Clind	Moxi
R5390	0.12	0.016	0.032	0.032	0.25	0.032	0.12
R5456	0.12	0.016	0.12	0.032	0.06	0.06	0.25
R5496	0.12	0.032	0.032	0.032	0.12	0.032	0.25
R5500	0.06	0.032	0.06	0.06	0.12	0.06	0.25
R5547	0.06	0.008	0.06	0.032	0.06	0.06	0.12
R5770	0.032	0.016	0.12	0.06	0.06	0.06	0.12
R5850	0.12	0.016	0.12	0.06	0.12	0.032	0.25
R5860	0.06	0.032	0.06	0.12	0.25	0.12	0.5
R5874	0.12	0.06	0.12	0.12	1	0.12	0.06
R5917	0.12	0.032	0.12	0.032	0.06	0.06	0.12
R5921	0.12	0.06	0.06	0.12	0.5	0.12	0.25
R5946	0.06	0.032	0.06	0.032	0.12	0.032	0.12
R5973	0.032	0.016	0.032	0.032	0.12	0.06	0.12
R6113	0.12	0.12	0.12	0.06	0.06	0.12	0.25
R6164	0.12	0.032	0.12	0.06	0.25	0.06	0.25
R6175	0.12	0.06	0.06	0.12	0.12	0.06	0.25
R6250	0.06	0.06	0.032	0.032	0.12	0.06	0.25
R6373	0.06	0.032	0.06	0.032	0.06	0.12	0.12
R6431	0.06	0.016	0.06	0.032	0.25	0.06	0.12
R7011	0.12	0.016	0.06	0.06	0.12	0.032	0.12
R7343	0.032	0.032	0.06	0.032	0.06	0.06	0.12
R7368	0.12	0.06	0.12	0.06	0.25	0.06	0.12
R7386	0.06	0.12	0.5	1	2	0.016	0.06
R7396	0.06	0.032	0.06	0.032	0.12	0.032	0.12
R7413	0.06	0.032	0.06	0.12	0.12	0.12	0.12
R7422	0.032	0.016	0.016	0.016	0.06	0.032	0.12
R7579	1	0.25	8	4	8	0.12	0.12
R7684	2	0.25	8	4	16	0.06	0.12
R7835	2	0.5	16	8	8	0.06	0.12
R7953	0.12	0.06	0.016	0.032	0.06	0.06	0.12

R8066	0.12	0.016	0.016	0.032	0.12	0.016	0.06
R8127	0.12	0.032	0.016	0.032	0.25	0.032	0.032
R8128	0.12	0.032	0.016	0.032	0.12	0.016	0.12
R8129	0.12	0.06	0.06	0.032	0.06	0.016	0.12
R8453	0.032	0.008	0.12	0.032	0.06	0.06	0.12
R8537	0.016	0.008	0.12	0.032	0.06	0.032	0.06
R8686	0.016	0.008	0.06	0.016	0.06	0.06	0.12
R8717	1	0.12	8	4	16	0.032	0.12
R9030	0.032	0.008	0.06	0.032	0.06	0.032	0.12
R9295	0.12	0.016	0.06	0.06	0.25	0.032	0.06
R9325	0.25	0.032	4	1	0.25	0.06	0.12
R9612	0.25	0.12	1	1	1	0.016	0.12
R9806	0.016	0.008	0.12	0.06	0.06	0.06	0.12
R10582	0.12	0.032	0.06	0.06	0.12	0.032	0.25
R10758	0.25	0.032	0.06	0.032	0.06	0.016	0.12
R40700	0.12	0.032	0.12	0.032	0.06	0.032	0.25
R70828	0.12	0.032	0.12	0.06	0.5	0.008	0.25
R77271	0.12	0.032	0.06	0.06	0.12	0.032	0.25
R79933	0.12	0.032	0.12	0.06	0.06	0.016	0.25
R80267	0.12	0.032	0.12	0.06	0.06	0.032	0.12
L80750	0.06	0.032	0.06	0.016	0.06	0.06	0.12
L80785	0.25	0.12	64	32	16	0.12	0.25
L80788	0.06	0.016	0.06	0.032	0.25	0.032	0.25
L85035	0.12	0.032	0.032	0.12	0.06	0.06	0.25
L85911	0.06	0.032	0.06	0.06	0.12	0.06	0.12
L85925	0.12	0.032	0.06	0.06	0.12	0.016	0.12
L85937	0.032	0.06	0.032	0.016	0.06	0.016	0.12
L85943	0.032	0.06	0.06	0.032	0.12	0.06	0.12
L89631	0.032	0.06	0.06	0.06	0.016	0.032	0.25
L89697	0.032	0.008	0.12	0.032	0.12	0.016	0.25
L89727	0.06	0.032	0.06	0.12	0.06	0.032	0.25
L89852	0.12	0.008	0.25	2	2	0.06	0.25
L89859	0.032	0.008	0.032	0.032	0.06	0.06	0.25
L89883	0.032	0.008	0.06	0.032	0.25	0.016	0.12
L89927	0.032	0.016	0.032	0.032	0.008	0.008	0.25
L89939	0.016	0.06	2	0.5	4	0.06	0.25
L7085059	0.12	0.016	0.008	0.032	0.032	0.06	0.12
L7085066	0.12	0.032	0.06	0.032	0.032	0.06	0.12
L7085268	0.06	0.016	0.06	0.12	0.06	0.06	0.25
L7090685	0.06	0.032	0.12	0.12	0.032	0.032	0.25
L7090700	0.12	0.12	0.25	0.5	0.5	0.032	0.25
L17	0.032	0.032	0.06	0.06	0.25	0.06	0.25
L22	0.032	0.016	0.06	0.032	0.06	0.032	0.25
L24	0.12	0.032	0.06	0.06	0.12	0.06	0.12
L29	0.06	0.06	0.032	0.032	0.06	0.06	0.25
L1	0.032	0.032	0.06	0.12	0.12	0.032	0.25

BPE 1	0.06	0.12	0.06	0.032	0.06	0.06	0.06
BPE 5	2	1	16	8	16	0.032	0.25
BPE 6	0.25	0.12	0.06	0.032	0.25	0.06	0.12
BPE 13	2	0.25	4	2	4	0.06	0.12
BPE 14	0.12	0.032	2	1	2	0.016	0.12
BPE 17	2	0.12	16	8	16	0.06	0.12
BPE 22	0.25	0.06	0.06	0.032	0.06	0.06	0.12
BPE 23	0.12	0.06	0.12	0.032	0.12	0.032	0.12
BPE 25	0.06	0.032	0.06	0.032	0.12	0.06	0.12
BPE 89	0.06	0.06	128	32	16	2	0.06
BPE 158	0.12	0.016	0.06	0.016	0.06	0.032	0.12
BPE 249	0.12	0.06	0.06	0.032	0.032	0.032	0.12
BPE 251	0.25	0.06	0.06	0.06	1	0.016	0.12
BPE 563	0.25	0.12	128	16	16	32	0.25
BPE 565	0.25	0.06	0.5	0.12	0.5	0.008	0.12
BPE 779	0.12	0.06	0.06	0.032	0.12	0.06	0.06
BPE 6080	0.06	0.06	0.06	0.032	0.12	0.032	0.12
A919	0.12	0.032	128	32	16	32	0.12
A2905	0.12	0.06	0.06	0.032	0.032	0.008	0.032
A7184	0.12	0.06	0.06	0.032	0.06	0.032	0.12
A8442	0.12	0.12	128	32	16	0.06	0.25
A11080	0.06	0.12	128	32	16	32	0.12
A12825	0.25	0.032	128	32	16	0.12	0.06
A13678	0.25	0.032	4	1	16	0.12	0.06
Strains	Levo	Cipro	Gemi	Linez	Amoxn	Amoxv	Farop
R5390	1	4	0.032	1	0.032	0.016	0.008
R5456	1	1	0.032	2	0.016	0.016	0.008
R5496	1	1	0.12	2	0.016	0.032	0.12
R5500	1	1	0.032	2	0.032	0.016	0.016
R5547	0.5	0.5	0.032	2	0.016	0.008	0.004
R5770	1	4	0.032	2	0.016	0.016	0.008
R5850	1	2	0.032	4	0.032	0.016	0.008
R5860	1	2	0.06	2	0.032	0.016	0.016
R5874	1	2	0.016	1	0.25	0.5	0.016
R5917	1	1	0.032	2	0.032	0.016	0.008
R5921	2	4	0.06	2	0.016	0.008	0.008
R5946	1	2	0.032	0.5	0.016	0.016	0.008
R5973	0.5	1	0.016	1	0.008	0.004	0.004
R6113	1	4	0.032	2	0.016	0.016	0.008
R6164	2	4	0.06	2	0.032	0.032	0.016
R6175	1	4	0.032	2	0.032	0.032	0.016
R6250	1	2	0.06	2	0.032	0.016	0.016
R6373	0.5	2	0.032	2	0.032	0.016	0.004

R6431	0.25	0.5	0.008	1	0.008	0.004	0.002
R7011	1	4	0.032	2	0.032	0.016	0.016
R7343	1	1	0.016	2	0.016	0.008	0.008
R7368	1	1	0.016	4	0.032	0.032	0.016
R7386	1	0.5	0.008	0.25	0.032	0.032	0.008
R7396	0.5	1	0.008	2	0.016	0.016	0.008
R7413	0.5	4	0.06	0.5	0.032	0.032	0.016
R7422	1	0.5	0.032	1	0.032	0.016	0.008
R7579	0.5	1	0.06	2	0.032	0.016	0.008
R7684	1	1	0.032	2	0.032	0.016	0.016
R7835	0.5	2	0.032	1	0.032	0.016	0.008
R7953	1	2	0.016	1	0.008	0.008	0.004
R8066	0.5	1	0.008	0.5	0.016	0.016	0.008
R8127	1	0.5	0.016	0.5	0.016	0.008	0.008
R8128	0.25	0.5	0.016	1	0.016	0.008	0.008
R8129	0.5	1	0.016	1	0.032	0.016	0.008
R8453	0.5	0.5	0.032	2	0.032	0.016	0.008
R8537	0.12	0.5	0.016	1	0.004	0.004	0.002
R8686	0.5	1	0.06	0.5	0.032	0.016	0.008
R8717	1	1	0.06	1	0.032	0.016	0.008
R9030	0.5	1	0.032	1	0.032	0.016	0.008
R9295	1	1	0.032	1	0.032	0.016	0.004
R9325	0.5	0.25	0.008	2	0.016	0.008	0.004
R9612	1	2	0.032	1	0.25	0.12	0.12
R9806	1	2	0.016	2	0.032	0.016	0.008
R10582	0.5	0.5	0.032	1	0.032	0.032	0.016
R10758	1	0.5	0.032	1	0.032	0.032	0.008
R40700	0.5	0.5	0.032	2	0.032	0.016	0.016
R70828	0.5	0.5	0.032	1	0.016	0.004	0.004
R77271	1	1	0.06	1	0.032	0.032	0.016
R79933	1	1	0.032	2	0.016	0.008	0.008
R80267	0.5	1	0.016	1	0.016	0.016	0.004
L80750	1	2	0.032	1	0.016	0.008	0.008
L80785	0.5	1	0.032	0.5	0.016	0.008	0.016
L80788	2	2	0.06	1	0.016	0.016	0.008
L85035	1	2	0.06	2	0.016	0.008	0.016
L85911	1	2	0.032	2	0.016	0.008	0.008
L85925	1	1	0.032	0.25	0.016	0.008	0.004
L85937	2	0.5	0.032	0.5	0.016	0.008	0.004
L85943	2	2	0.032	0.5	0.016	0.008	0.008
L89631	2	2	0.032	0.5	0.032	0.032	0.016
L89697	2	0.25	0.032	0.25	0.032	0.016	0.016
L89727	2	2	0.032	2	0.016	0.008	0.008
L89852	1	4	0.12	0.25	0.016	0.008	0.12
L89859	1	2	0.016	1	0.016	0.008	0.016
L89883	2	0.5	0.008	0.25	0.032	0.032	0.008

L89927	2	2	0.008	0.12	0.016	0.008	0.004
L89939	1	0.5	0.016	0.12	0.016	0.008	0.008
L7085059	2	2	0.008	1	0.008	0.008	0.002
L7085066	0.12	2	0.008	1	0.008	0.008	0.002
L7085268	1	4	0.06	2	0.016	0.008	0.016
L7090685	1	2	0.06	2	0.016	0.008	0.008
L7090700	1	4	0.06	1	0.5	0.25	0.12
L17	0.5	2	0.032	0.5	0.008	0.008	0.004
L22	2	2	0.06	0.5	0.008	0.008	0.004
L24	2	1	0.032	1	0.008	0.008	0.008
L29	1	1	0.032	2	0.032	0.06	0.008
L1	1	1	0.032	1	0.008	0.008	0.008
BPE 1	1	1	0.016	0.5	0.016	0.008	0.008
BPE 5	1	0.25	0.016	1	0.032	0.016	0.008
BPE 6	1	0.5	0.016	2	1	2	0.5
BPE 13	1	0.5	0.032	0.25	0.032	0.016	0.016
BPE 14	1	0.5	0.032	2	0.12	0.12	0.06
BPE 17	2	4	0.016	1	0.032	0.016	0.12
BPE 22	1	0.5	0.016	2	0.06	0.016	0.008
BPE 23	2	2	0.016	2	2	2	1
BPE 25	0.5	2	0.032	1	0.016	0.016	0.016
BPE 89	0.5	1	0.016	0.25	0.5	0.5	0.25
BPE 158	1	2	0.016	0.5	2	1	0.5
BPE 249	0.5	1	0.016	2	2	1	0.25
BPE 251	1	8	0.12	1	1	1	0.25
BPE 563	1	1	0.016	1	0.5	1	0.12
BPE 565	2	4	0.032	1	0.5	0.5	0.12
BPE 779	0.5	0.5	0.016	1	1	1	0.5
BPE 6080	1	4	0.032	1	0.25	0.25	0.25
A919	1	1	0.032	0.5	0.06	0.032	0.06
A2905	0.25	0.25	0.016	2	0.5	1	0.12
A7184	0.5	0.5	0.032	1	0.008	0.032	0.032
A8442	0.5	2	0.032	0.25	0.06	0.06	0.06
A11080	2	8	0.06	1	1	1	0.25
A12825	0.5	0.25	0.032	4	0.12	0.06	0.06
A13678	0.5	0.032	0.032	2	0.12	0.06	0.12

Moraxella catarrhalis MICs

Strains	Telith CO_2	Telith air	Ery	Clar	Azith	Clind	Moxi
R1227	0.06	0.06	0.12	0.06	0.032	2	0.016
R7504	0.12	0.06	0.25	0.12	0.06	2	0.06
R7544	0.12	0.06	0.12	0.06	0.06	2	0.06
R7829	0.12	0.06	0.25	0.12	0.06	2	0.06
R7962	0.25	0.12	0.12	0.12	0.06	2	0.06
R8041	0.12	0.12	0.25	0.25	0.06	2	0.06
R10699	0.12	0.06	0.25	0.06	0.06	2	0.06
R10703	0.06	0.06	0.12	0.12	0.06	2	0.06
R10836	0.12	0.06	0.25	0.12	0.06	2	0.06
R10848	0.12	0.06	0.12	0.12	0.032	2	0.06
R10853	0.12	0.06	0.25	0.12	0.06	4	0.06
R10855	0.12	0.06	0.12	0.12	0.06	2	0.032
R10863	0.06	0.06	0.12	0.06	0.06	2	0.06
R11871	0.06	0.032	0.12	0.06	0.06	2	0.06
R12039	0.06	0.032	0.12	0.12	0.06	2	0.032
R12194	0.032	0.032	0.12	0.06	0.032	2	0.032
R12234	0.12	0.06	0.12	0.12	0.06	2	0.06
R12774	0.12	0.06	0.12	0.12	0.032	2	0.06
R12797	0.12	0.12	0.12	0.06	0.06	2	0.06
R12830	0.12	0.06	0.12	0.06	0.25	2	0.06
R13703	0.06	0.06	0.12	0.06	0.06	1	0.032
BME 1	0.06	0.06	0.25	0.06	0.06	4	0.032
BME 2	0.12	0.032	0.12	0.032	0.06	1	0.06
BME 3	0.06	0.06	0.12	0.06	0.032	2	0.032
BME 5	0.12	0.06	0.25	0.12	0.25	2	0.032
BME 7	0.12	0.06	0.12	0.12	0.06	2	0.032
BME 8	0.12	0.06	0.25	0.12	0.06	4	0.06
BME 9	0.12	0.12	0.5	0.25	0.12	4	0.06
BME 10	0.06	0.06	0.12	0.12	0.06	4	0.032
BME 12	0.12	0.06	0.12	0.12	0.032	2	0.06
BME 21	0.12	0.06	0.25	0.12	0.06	2	0.06
BME 22	0.06	0.06	0.25	0.12	0.06	2	0.06
BME 24	0.06	0.06	0.25	0.12	0.06	2	0.06
BME 25	0.06	0.06	0.25	0.25	0.06	2	0.032
BME 26	0.06	0.06	0.25	0.12	0.06	2	0.06
BME 34	0.12	0.06	0.25	0.12	0.06	4	0.06
BME 37	0.06	0.06	0.12	0.12	0.06	2	0.06
BME 38	0.06	0.032	0.12	0.06	0.032	2	0.06
BME 39	0.06	0.06	0.25	0.12	0.06	2	0.06
BME 40	0.06	0.06	0.25	0.12	0.032	1	0.032
BME 41	0.12	0.06	0.25	0.06	0.032	1	0.032
BME 42	0.06	0.06	0.25	0.12	0.06	2	0.06

BME 43	0.06	0.06	0.25	0.12	0.032	2	0.06
BME 44	0.25	0.12	0.25	0.12	0.12	2	0.06
BME 45	0.06	0.06	0.12	0.06	0.032	2	0.032
BME 46	0.12	0.06	0.25	0.12	0.06	2	0.032
LM75591	1	0.5	1	0.25	0.25	8	0.032
LM75608	0.06	0.06	0.12	0.12	0.06	8	0.032
LM75735	0.25	0.12	0.25	0.12	0.06	1	0.06
LM75751	0.12	0.12	0.25	0.12	0.12	2	0.06
LM75758	0.12	0.06	0.12	0.032	0.06	2	0.06
LM80738	0.12	0.06	0.25	0.12	0.12	2	0.06
LM80752	0.12	0.06	0.12	0.06	0.032	2	0.06
LM80776	0.12	0.12	0.25	0.06	0.06	4	0.06
LM82359	0.12	0.12	0.12	0.06	0.06	2	0.06
LM83095	0.25	0.12	0.25	0.12	0.06	4	0.06
LM83096	0.25	0.12	0.25	0.12	0.06	1	0.06
LM83106	0.25	0.12	0.25	0.12	0.12	2	0.06
LM83760	0.25	0.12	0.25	0.12	0.06	2	0.06
LM83791	0.25	0.25	0.25	0.12	0.06	4	0.06
LM85824	0.25	0.25	0.25	0.12	0.12	2	0.06
LM85899	0.25	0.12	0.25	0.12	0.06	2	0.032
LM85919	0.12	0.06	0.12	0.06	0.12	4	0.016
LM86000	0.12	0.06	0.12	0.06	0.06	2	0.06
LM89310	0.25	0.12	0.12	0.12	0.06	2	0.06
LM89517	0.25	0.25	0.25	0.12	0.06	1	0.06
LM89539	0.12	0.06	0.25	0.06	0.12	4	0.12
LM89724	0.12	0.06	0.06	0.032	0.06	4	0.06
LM89727	0.25	0.25	0.25	0.12	0.12	1	0.06
LM89784	0.06	0.032	0.12	0.032	0.032	2	0.06
LM89798	0.25	0.12	0.25	0.06	0.06	2	0.032
LM89848	0.12	0.06	0.25	0.12	0.06	2	0.06
LM89886	0.12	0.12	0.25	0.06	0.06	2	0.06
LM7085099	0.12	0.12	0.25	0.12	0.25	8	0.06
LM7085273	0.12	0.12	0.25	0.06	0.12	4	0.06
LM48	0.12	0.12	0.25	0.06	0.06	2	0.06
BMW 1	0.25	0.06	0.12	0.12	0.06	2	0.12
BMW 2	0.12	0.06	0.12	0.06	0.06	2	0.06
BMW 3	0.12	0.06	0.12	0.06	0.06	1	0.06
BMW 4	0.12	0.06	0.12	0.06	0.06	2	0.06
BMW 5	0.12	0.06	0.12	0.06	0.06	4	0.016
BMW 6	0.25	0.06	0.25	0.12	0.06	2	0.06
BMW 7	0.25	0.06	0.25	0.06	0.06	2	0.06
BMW 8	0.12	0.12	0.25	0.06	0.06	2	0.032
BMW 9	0.12	0.12	0.25	0.12	0.06	2	0.06
BMW 10	0.25	0.12	0.25	0.12	0.06	2	0.06
BMW 12	0.12	0.06	0.25	0.12	0.06	2	0.06
BMW 15	0.12	0.12	0.25	0.06	0.06	2	0.06

BMW 16	0.25	0.06	0.12	0.12	0.032	1	0.06
BMW 18	0.12	0.032	0.12	0.06	0.032	1	0.06
BMW 20	0.25	0.06	0.12	0.06	0.06	2	0.06
BMB 15	0.25	0.12	0.25	0.12	0.06	4	0.06
BMB 16	0.25	0.12	0.12	0.06	0.06	2	0.06
BMB 26	0.25	0.12	0.12	0.06	0.032	1	0.06
BMB 27	0.25	0.12	0.25	0.06	0.06	2	0.06
BMB 30	0.25	0.06	0.25	0.12	0.06	1	0.06
BMB 34	0.12	0.12	0.12	0.12	0.06	2	0.032
BMB 37	0.12	0.06	0.12	0.12	0.032	1	0.032
BMB 38	0.25	0.12	0.12	0.12	0.06	2	0.032
BMB 39	0.25	0.12	0.12	0.12	0.032	1	0.032
Strains	Levo	Cipro	Gemi	Linez	Amoxn	Amoxv	Farop
R1227	0.032	0.016	0.002	4	0.5	0.008	0.12
R7504	0.032	0.032	0.008	4	2	0.06	0.25
R7544	0.032	0.032	0.004	4	4	0.12	0.5
R7829	0.06	0.032	0.016	4	2	0.06	0.25
R7962	0.032	0.032	0.008	4	0.5	0.008	0.06
R8041	0.032	0.032	0.008	4	2	0.25	0.5
R10699	0.06	0.032	0.016	4	0.25	0.004	0.032
R10703	0.032	0.016	0.008	2	1	0.06	0.25
R10836	0.032	0.032	0.016	4	2	0.06	0.25
R10848	0.032	0.032	0.016	4	0.5	0.008	0.032
R10853	0.06	0.032	0.016	8	0.5	0.004	0.032
R10855	0.032	0.016	0.008	4	0.25	0.004	0.032
R10863	0.032	0.032	0.016	4	1	0.008	0.06
R11871	0.032	0.032	0.008	2	0.5	0.004	0.032
R12039	0.032	0.032	0.004	4	0.016	0.002	0.032
R12194	0.032	0.032	0.008	2	0.016	0.002	0.032
R12234	0.032	0.032	0.008	4	2	0.06	0.25
R12774	0.032	0.032	0.008	4	2	0.12	0.25
R12797	0.032	0.032	0.016	4	2	0.06	0.25
R12830	0.06	0.032	0.008	4	0.12	0.002	0.06
R13703	0.032	0.032	0.008	4	1	0.06	0.5
BME 1	0.06	0.032	0.016	8	1	0.004	0.032
BME 2	0.032	0.032	0.008	4	4	0.032	0.25
BME 3	0.032	0.032	0.008	4	2	0.25	1
BME 5	0.032	0.032	0.016	4	2	0.032	0.12
BME 7	0.032	0.016	0.008	4	0.12	0.002	0.032
BME 8	0.06	0.06	0.016	8	0.5	0.008	0.06
BME 9	0.06	0.032	0.016	4	4	0.25	0.5
BME 10	0.032	0.032	0.008	4	1	0.016	0.06
BME 12	0.06	0.032	0.016	4	4	0.12	0.5
BME 21	0.032	0.032	0.008	4	8	0.25	1

BME 22	0.032	0.06	0.008	4	4	0.25	1
BME 24	0.06	0.06	0.008	4	0.5	0.008	0.06
BME 25	0.032	0.032	0.008	8	4	0.25	0.5
BME 26	0.06	0.06	0.016	8	1	0.008	0.06
BME 34	0.032	0.032	0.008	8	1	0.008	0.06
BME 37	0.032	0.032	0.016	4	2	0.12	0.25
BME 38	0.06	0.032	0.008	4	0.5	0.016	0.06
BME 39	0.06	0.032	0.016	4	0.5	0.016	0.06
BME 40	0.032	0.032	0.016	4	0.5	0.002	0.032
BME 41	0.032	0.032	0.008	4	4	0.12	1
BME 42	0.06	0.032	0.016	4	0.25	0.004	0.06
BME 43	0.06	0.032	0.016	4	4	0.25	1
BME 44	0.12	0.06	0.016	4	4	0.032	0.25
BME 45	0.032	0.032	0.016	4	0.5	0.002	0.032
BME 46	0.032	0.032	0.016	4	4	0.12	0.5
LM75591	0.06	0.016	0.008	4	2	0.032	0.25
LM75608	0.032	0.032	0.004	8	1	0.032	0.12
LM75735	0.032	0.032	0.008	4	4	0.12	1
LM75751	0.032	0.016	0.008	4	4	0.032	0.5
LM75758	0.032	0.032	0.016	4	0.016	0.002	0.06
LM80738	0.06	0.016	0.008	4	0.5	0.002	0.06
LM80752	0.032	0.032	0.008	4	2	0.06	0.25
LM80776	0.032	0.032	0.016	4	4	0.12	0.5
LM82359	0.032	0.032	0.008	4	2	0.032	0.25
LM83095	0.06	0.032	0.008	4	0.5	0.004	0.06
LM83096	0.032	0.032	0.016	4	4	0.12	1
LM83106	0.032	0.032	0.016	4	0.5	0.004	0.06
LM83760	0.032	0.032	0.008	4	4	0.12	0.5
LM83791	0.06	0.032	0.016	8	4	0.06	0.25
LM85824	0.032	0.032	0.008	4	4	0.12	0.5
LM85899	0.032	0.016	0.004	4	1	0.004	0.25
LM85919	0.016	0.016	0.002	4	1	0.032	0.25
LM86000	0.032	0.032	0.008	8	0.5	0.004	0.032
LM89310	0.06	0.06	0.016	4	1	0.06	0.5
LM89517	0.06	0.032	0.016	4	4	0.25	1
LM89539	0.06	0.032	0.016	2	0.5	0.002	0.06
LM89724	0.06	0.06	0.016	4	1	0.06	0.25
LM89727	0.06	0.06	0.016	4	2	0.12	1
LM89784	0.06	0.032	0.016	4	2	0.06	1
LM89798	0.032	0.032	0.008	4	1	0.008	0.06
LM89848	0.06	0.032	0.016	4	4	0.12	1
LM89886	0.032	0.032	0.016	4	2	0.004	0.25
LM7085099	0.06	0.032	0.016	4	1	0.06	0.25
LM7085273	0.06	0.032	0.016	4	0.016	0.004	0.06
LM48	0.06	0.032	0.016	4	2	0.06	0.5
BMW 1	0.032	0.032	0.016	4	4	0.06	0.5

BMW 2	0.032	0.06	0.016	2	0.06	0.06	0.25
BMW 3	0.016	0.06	0.016	2	0.5	0.004	0.032
BMW 4	0.032	0.06	0.016	4	0.06	0.032	0.12
BMW 5	0.016	0.016	0.002	2	2	0.06	0.12
BMW 6	0.032	0.032	0.016	2	4	0.032	0.5
BMW 7	0.032	0.032	0.016	4	2	0.032	0.12
BMW 8	0.032	0.032	0.008	2	2	0.12	0.25
BMW 9	0.032	0.06	0.016	2	0.25	0.008	0.06
BMW 10	0.032	0.032	0.016	2	0.016	0.008	0.06
BMW 12	0.06	0.06	0.016	8	8	0.016	0.5
BMW 15	0.032	0.032	0.016	4	0.5	0.008	0.06
BMW 16	0.016	0.032	0.008	2	4	0.032	0.25
BMW 18	0.032	0.032	0.008	2	0.25	0.008	0.032
BMW 20	0.032	0.06	0.016	2	0.25	0.008	0.06
BMB 15	0.032	0.032	0.008	4	0.5	0.004	0.06
BMB 16	0.032	0.032	0.008	4	1	0.004	0.06
BMB 26	0.016	0.032	0.008	2	4	0.25	0.5
BMB 27	0.032	0.032	0.016	4	1	0.008	0.06
BMB 30	0.032	0.06	0.008	2	1	0.002	0.032
BMB 34	0.032	0.032	0.004	4	4	0.12	0.25
BMB 37	0.032	0.032	0.004	2	4	0.06	0.5
BMB 38	0.032	0.032	0.004	2	0.5	0.002	0.032
BMB 39	0.016	0.016	0.008	2	0.25	0.002	0.032

Haemophilus influenzae MICs

Strains	Telith CO_2	Telith air	Ery	Clar	Azith	Clind	Moxi
R5580	4	1	4	16	1	4	0.008
R7410	2	0.5	4	8	2	4	0.016
R7434	2	1	4	16	2	4	0.016
R7456	4	2	1	8	4	1	0.016
R7466	2	0.5	2	4	0.5	1	0.008
R7491	8	2	8	8	1	2	0.016
R7508	2	0.5	2	4	0.5	4	0.008
R7517	2	2	8	1	2	8	0.016
R7544	0.12	0.12	0.25	0.12	0.25	2	0.12
R7832	2	0.5	2	8	1	4	0.016
R7859	8	4	8	8	1	1	0.016
R7953	0.008	1	4	8	1	2	0.008
R7974	4	1	4	8	2	8	0.016
R8013	4	1	2	4	1	1	0.032
R8189	4	4	1	8	2	1	0.016
R8280	8	4	1	16	4	1	0.032
R8687	2	1	4	8	2	2	0.016
R8701	2	1	2	8	1	8	0.016
R8714	2	1	8	8	1	8	0.008
R8923	1	1	2	4	1	2	0.004
R8953	1	0.25	1	4	0.5	1	0.008
R9006	2	1	2	4	0.5	1	0.016
R9027	2	1	4	8	1	4	0.016
R9030	2	0.5	2	2	0.5	8	0.016
R9032	1	0.25	4	8	1	2	0.008
R9033	2	0.5	4	4	1	2	0.016
R9270	2	1	4	16	1	4	0.016
R9289	2	0.5	4	4	1	8	0.008
R9300	1	0.5	2	8	2	8	0.008
R9309	4	4	1	16	8	32	0.016
R9318	1	0.5	2	4	1	4	0.008
R9325	2	1	1	16	2	1	0.016
R9341	4	0.5	4	8	1	8	0.016
R9359	2	0.25	4	8	1	4	0.004
R9360	2	1	8	4	0.5	2	0.004
R9365	2	0.5	2	2	0.5	2	0.016
R9506	2	1	8	16	4	1	0.016
R9575	0.5	0.25	4	4	0.5	2	0.032
R9601	2	0.5	8	8	2	2	0.008
R9846	4	2	1	16	1	1	0.016
R63403	1	0.5	4	4	1	4	0.004
R71113	2	0.5	4	4	1	1	0.008

BHG 1	2	1	4	8	1	8	0.016
BHG 2	0.06	0.032	0.25	0.06	0.06	1	0.004
BHG 3	0.5	0.5	2	4	0.5	4	0.004
BHG 4	1	0.5	4	4	0.5	2	0.016
BHG 6	4	2	1	16	2	1	0.016
BHG 7	2	0.5	4	8	0.5	8	0.016
BHG 9	0.5	0.5	0.5	2	1	1	0.004
BHG 13	2	0.5	2	4	0.5	2	0.008
BHG 14	8	2	4	8	1	8	0.008
BHG 15	1	0.5	2	2	0.5	2	0.008
BHG 17	1	1	4	2	0.5	1	0.008
BHG 18	2	1	8	4	1	1	0.016
BHG 19	2	0.5	2	2	2	2	0.016
BHG 20	1	1	4	2	0.5	0.25	0.016
BHG 21	2	1	2	2	0.5	0.5	0.008
BHG 22	2	0.5	4	8	1	1	0.016
BHG 23	2	2	8	8	2	4	0.004
BHG 24	2	0.5	4	8	0.5	8	0.016
BHG 25	2	1	4	8	0.5	4	0.016
BHG 26	0.12	0.25	0.5	1	0.12	1	0.016
BHG 27	2	2	4	8	1	8	0.016
BHG 29	2	1	4	8	1	8	0.016
BHG 30	1	1	2	4	0.25	1	0.016
BHG 32	1	1	2	1	0.25	1	0.008
BHG 34	1	1	4	8	1	8	0.004
BHG 35	2	2	8	8	1	4	0.016
BHG 37	4	1	2	4	0.5	2	0.016
BHG 38	1	1	4	4	0.5	2	0.016
BHG 40	4	2	8	8	4	1	0.008
BHG 42	1	0.5	2	2	0.5	2	0.016
BHG 43	1	0.5	4	2	1	4	0.016
BHG 44	0.5	0.25	2	0.5	0.25	1	0.016
BHG 45	2	1	4	16	1	4	0.016
BHG 46	2	1	4	8	4	1	0.004
BHG 47	0.5	0.25	2	4	1	8	0.008
BHG 48	0.5	0.25	8	16	2	4	0.032
BHG 49	2	0.5	4	4	2	2	0.016
BHG 50	2	0.5	4	8	2	2	0.016
BHG 51	2	1	4	4	1	4	0.032
BHG 55	4	2	1	16	2	32	0.016
BHG 56	0.06	0.032	0.12	0.06	0.12	0.032	0.12
BHG 57	1	0.5	2	2	0.5	1	0.016
BHG 58	2	1	2	4	1	2	0.032
BHG 62	0.5	0.5	2	2	0.5	2	0.016
BHG 65	4	4	8	8	1	4	0.032
BHG 66	2	0.2	0.5	2	0.5	1	0.016

BHG 70	4	4	8	8	2	4	0.016
BHG 72	1	0.5	2	4	0.5	4	0.016
BHG 74	1	1	4	4	1	4	0.008
BHG 75	0.5	0.25	2	2	0.5	1	0.008
BHG 77	4	2	4	8	1	2	0.016
BHG 78	0.5	0.12	2	1	0.2	2	0.016
BHG 80	2	1	4	4	2	4	0.016
BHG 81	2	1	4	4	2	4	0.016
BHG 82	1	0.5	2	2	0.2	2	0.004
BHG 89	1	1	4	8	1	8	0.016
BHG 92	1	0.5	2	4	0.5	4	0.008
BHG 94	4	2	4	8	2	4	0.008
Strains	Levo	Cipro	Gemi	Linez	Amoxn	Amoxv	Farop
R5580	0.008	0.008	0.002	1	1	0.5	0.5
R7410	0.008	0.008	0.002	8	0.5	0.5	0.5
R7434	0.008	0.008	0.002	1	2	1	4
R7456	0.016	0.008	0.002	1	1	0.5	1
R7466	0.008	0.004	0.002	1	2	0.1	0.12
R7491	0.01	0.008	0.002	1	2	0.5	0.2
R7508	0.008	0.008	0.002	1	0.5	0.5	0.5
R7517	0.008	0.008	0.002	1	0.5	0.5	1
R7544	0.12	0.032	0.016	4	4	0.06	2
R7832	0.008	0.008	0.002	8	1	0.5	0.5
R7859	0.008	0.008	0.002	32	1	0.5	2
R7953	0.008	0.002	0.002	1	0.5	0.5	0.5
R7974	0.016	0.008	0.002	4	0.5	0.25	0.25
R8013	0.016	0.016	0.004	64	4	0.5	0.5
R8189	0.016	0.016	0.004	32	1	2	1
R8280	0.016	0.008	0.008	8	32	1	2
R8687	0.016	0.016	0.004	4	0.5	0.5	1
R8701	0.016	0.008	0.002	4	0.5	0.5	1
R8714	0.008	0.008	0.002	1	0.5	0.25	0.5
R8923	0.016	0.008	0.002	8	0.2	0.1	0.25
R8953	0.008	0.004	0.002	2	0.5	0.5	0.25
R9006	0.008	0.008	0.002	4	1	1	1
R9027	0.008	0.008	0.002	1	0.5	0.5	0.5
R9030	0.016	0.008	0.002	4	0.5	0.5	0.5
R9032	0.016	0.008	0.002	8	2	0.5	0.5
R9033	0.008	0.008	0.002	1	0.5	0.5	1
R9270	0.008	0.004	0.002	32	0.5	0.5	1
R9289	0.016	0.008	0.002	4	1	2	0.5
R9300	0.008	0.008	0.004	4	2	2	0.5
R9309	0.016	0.008	0.002	32	1	1	1
R9318	0.008	0.004	0.004	8	0.5	0.25	0.25

R9325	0.016	0.016	0.004	1	2	0.25	0.1
R9341	0.016	0.008	0.004	4	1	0.5	0.5
R9359	0.008	0.008	0.002	8	0.25	8	0.25
R9360	0.008	0.004	0.002	8	0.25	0.25	0.5
R9365	0.016	0.016	0.004	32	0.25	0.25	0.5
R9506	0.016	0.008	0.002	32	0.5	0.5	1
R9575	0.016	0.032	0.004	1	1	1	1
R9601	0.008	0.008	0.002	1	0.5	0.5	1
R9846	0.016	0.008	0.002	1	2	0.5	0.25
R63403	0.008	0.004	0.002	8	0.5	0.1	0.5
R71113	0.008	0.008	0.002	8	0.5	0.25	0.5
BHG 1	0.008	0.008	0.002	8	0.5	0.25	0.5
BHG 2	0.008	0.004	0.002	4	0.032	0.016	0.06
BHG 3	0.008	0.008	0.002	4	4	0.5	0.5
BHG 4	0.016	0.008	0.004	4	0.5	0.5	1
BHG 6	0.008	0.008	0.004	8	0.5	0.06	0.12
BHG 7	0.008	0.008	0.002	8	8	0.2	0.5
BHG 9	0.016	0.008	0.002	4	0.1	0.5	0.5
BHG 13	0.008	0.008	0.002	4	0.5	0.5	0.5
BHG 14	0.008	0.008	0.004	1	0.25	0.25	0.25
BHG 15	0.008	0.008	0.002	8	0.5	0.25	0.25
BHG 17	0.008	0.008	0.002	4	1	1	0.5
BHG 18	0.016	0.008	0.002	1	0.5	0.5	1
BHG 19	0.016	0.004	0.002	8	0.5	0.5	0.5
BHG 20	0.008	0.004	0.004	4	1	0.5	2
BHG 21	0.008	0.004	0.002	4	2	1	2
BHG 22	0.008	0.004	0.002	4	1	0.5	0.5
BHG 23	0.008	0.002	0.002	8	0.5	0.5	0.5
BHG 24	0.016	0.008	0.002	4	0.5	0.5	0.5
BHG 25	0.016	0.016	0.002	4	0.25	0.5	0.25
BHG 26	0.016	0.016	0.002	4	8	0.2	0.5
BHG 27	0.016	0.008	0.004	8	8	0.25	0.25
BHG 29	0.016	0.008	0.002	1	2	2	4
BHG 30	0.016	0.016	0.004	8	0.5	0.5	1
BHG 32	0.016	0.008	0.002	4	0.25	0.1	0.25
BHG 34	0.008	0.004	0.004	1	0.5	1	0.5
BHG 35	0.016	0.008	0.002	8	1	1	1
BHG 37	0.016	0.008	0.002	4	0.5	0.12	0.25
BHG 38	0.016	0.008	0.002	1	0.5	0.2	0.5
BHG 40	0.016	0.008	0.002	8	0.5	2	4
BHG 42	0.016	0.016	0.002	8	0.2	0.25	0.5
BHG 43	0.016	0.008	0.002	8	0.5	0.5	0.5
BHG 44	0.016	0.008	0.002	4	0.5	1	0.5
BHG 45	0.016	0.008	0.002	4	8	0.25	0.5
BHG 46	0.008	0.004	0.002	1	0.5	1	0.5
BHG 47	0.016	0.008	0.002	8	0.5	0.5	0.25

BHG 48	0.016	0.032	0.004	1	0.12	0.25	0.25
BHG 49	0.008	0.008	0.002	2	0.5	0.5	0.5
BHG 50	0.008	0.008	0.002	2	0.5	0.5	0.5
BHG 51	0.016	0.008	0.002	8	0.5	1	0.25
BHG 55	0.016	0.008	0.002	32	0.5	0.5	1
BHG 56	0.5	0.5	0.12	0.5	0.032	0.016	0.06
BHG 57	0.016	0.008	0.004	4	0.25	0.25	0.5
BHG 58	0.032	0.016	0.004	8	0.5	0.5	0.5
BHG 62	0.016	0.004	0.002	8	0.5	0.5	0.25
BHG 65	0.12	0.12	0.032	32	4	0.5	2
BHG 66	0.016	0.004	0.002	2	0.5	0.25	1
BHG 70	0.016	0.008	0.004	1	4	0.25	0.5
BHG 72	0.016	0.008	0.002	4	0.25	0.12	0.06
BHG 74	0.008	0.008	0.002	4	4	0.25	0.25
BHG 75	0.008	0.008	0.002	8	0.5	0.25	0.5
BHG 77	0.016	0.008	0.004	4	0.5	0.5	0.5
BHG 78	0.016	0.008	0.002	4	0.25	0.25	0.12
BHG 80	0.016	0.01	0.002	4	0.5	0.25	0.5
BHG 81	0.016	0.008	0.004	4	0.5	0.25	0.5
BHG 82	0.008	0.004	0.002	1	0.25	0.5	0.25
BHG 89	0.016	0.008	0.002	8	0.5	0.25	0.5
BHG 92	0.008	0.008	0.002	1	0.5	0.25	0.12
BHG 94	0.016	0.008	0.002	4	0.5	0.25	0.5

Appendix 2

Macrolide Resistant Strains MICs

Strains	Telith	Ery	Clar	Azith	Clind	Country of origin
5649	0.5	8	4	32	0.12	USA
5970	0.06	>64	>64	>64	>8	USA
950672	0.5	16	16	16	0.12	Belgium
950673	0.12	>64	>64	>64	>8	Belgium
950871	0.032	>64	>64	>64	>8	Belgium
950881	0.5	16	8	32	0.06	Belgium
02J1095	0.06	>64	>64	>64	>8	USA
02J1175	0.5	32	16	64	0.12	USA
59C072	0.25	16	16	32	0.12	Belgium
59C093	0.12	>64	>64	>64	>8	Belgium
59C113	0.06	16	16	>64	>8	Belgium
59C115	0.06	>64	>64	>64	>8	Belgium
917	0.06	>64	>64	>64	>32	Italy
931	0.06	64	64	>64	>32	Italy
950	2	>64	>64	>64	>32	Italy
951	2	>64	>64	>64	>32	Italy
1010	2	>64	>64	>64	>32	Italy
1035	0.5	16	4	>64	>32	Italy
6662	0.25	>16	>16	>32	>16	Canada

6719	0.032	>16	16	>32	2	Canada
6823	0.032	4	1	8	0.25	Canada
6926	0.032	>16	>16	>32	>16	Canada
6942	0.12	>16	16	>32	>16	Canada
7218	0.12	>16	>16	>32	>16	Canada
7332	0.25	4	2	8	0.12	Canada
7380	0.12	8	4	16	0.12	Canada
7488	0.06	>16	>16	>32	>16	Canada
7642	0.5	8	4	16	0.25	Canada
8185	0.12	>16	>16	>32	>16	Canada

Appendix 3

Sequence alignment of *ermB* genes

```
pAM77     taataggaattgaagttaaattagatgctaaaaatttgt
pAMb1     taataggaattgaagttaaattagatgctaaaaatttgt
Tn917     taataggaattgaagttaaattagatgctaaaaatttgt
Tn1545    taataggaattgaagttaaattagatgctaaaaatttgt
02J1095   TAATAGGAATTGAAGTTAAATTAGATGCTAAAAATTTGT
JIII8     TAATAGGAATTGAAGTTAAATTAGATGCTAAAAATTTGT

                        RBS 1                    Control
pAM77     aattaagaaggagggattcgtcatgttggtattccaaat
pAMb1     aattaag
Tn917     aattaagaaggagggattcgtcatgttggtattccaaat
Tn1545    aattaagaaggagggattcgtcatgttggtattccaaat
02J1095   AATTAAGAAGGAGGGATTCGTCATGTTGGTATTCCAAAT
JIII8     AATTAAGAAGGAGGGATT

             peptide
pAM77     gcgtaatgtagataaaacatctactattttgaaacagac
Tn917     gcgtaatgtagataaaacatctactgttttgaaacagac
Tn1545    gcgtaatgtagataaaacatctactgttttgaaacagac
02J1095   GCGTAATGTAGATAAAACATCTACTGTTTTGAAACAGAC

pAM77     taaaaacagtgattacgtagataaatacgttagattaat
Tn917     taaaaacagtgattacgcagataaataaatacgttagat
Tn1545    taaaaacagtgattacgcagataaataaatacgttagat
02J1095   TAAAAACAGTGATTACGCAGATAAATAAATACGTTAGAT

pAM77        tcctaccagtgactaatcttatgacttttaaaca
Tn917     taattcctaccagtgactaatcttatgacttttaaaca
Tn1545    taattcctaccagtgactaatcttatgacttttaaaca
02J1095   TAATTCCTACCAGTGACTAATCTTATGACTTTTTAAACA

pAM77     gataactaaaattacaaacaaatcgtttaacttctgtat
Tn917     gataactaaaattacaaacaaatcgtttaacttctgtat
Tn1545    gataactaaaattacaaacaaatcgtttaacttctgtat
02J1095   GATAACTAAAATTACAAACAAATCGTTTAACTTCTGTAT

                                    RBS 2      ermB
pAM77     ttgtttatagatgta tcacttcaggagtgattacatga
pAMb1                             aggagtgattacatga
Tn917     ttatttatagatgtaatcacttcaggagtgattacatga
Tn1545    ttatttacagatgtaatcacttcaggagtaattacatga
02J1095   TTGTTTATAGATGTAATCACTTCAGGAGAGATTACATGA
JIII8                                     ACATGA
```

	methylase gene
pAM77	aaaaaaatataaaatattctcaaaacttttaacg
pAMb1	acaaaaatataaaatattctcaaaacttttaacg
Tn917	acaaaaatataaaatattctcaaaacttttaacg
Tn1545	acaaaaatataaaatattctcaaaacttttaacg
02J1095	ACAAAAATATAAAATATTCTCAAAACTTTTTAACG
JIII8	ACAAAAATATAAAATATTCTCAAAACTTTTTAACG

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LETTER

Culturing *Chlamydophila pneumoniae*

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Chlamydophila pneumoniae (formerly *Chlamydia pneumoniae*) is an intracellular pathogen responsible for respiratory tract infection. A recent British survey of the microbial aetiology of community-acquired pneumonia (CAP) indicated that *C. pneumonia* is responsible for 13% of CAP and is the second highest bacterial cause of CAP [1]. It has been associated with bronchitis, pharyngitis, sinusitis, myocarditis, endocarditis and coronary artery disease [2].

In order to treat *C. pneumoniae* infections it is first essential to know the efficacy of the available antimicrobial agents against this pathogen. Minimum inhibitory concentration (MIC) tests are used to investigate the *in vitro* capabilities of antimicrobial agents on bacteria. Therefore, MIC testing of *C. pneumoniae* needs to be carried out before an antimicrobial agent is used to treat the infection. Culturing of *C. pneumoniae* must occur before MIC tests can be performed. *C. pneumoniae* is known to be very difficult to culture and is far more difficult than other chlamydial species [2]. Many methods of culturing *C. pneumoniae* have been proposed with differing cell lines, centrifugation conditions and incubation times. There is no standard method for culturing *C. pneumoniae* nor for testing the MIC of antimicrobial agents against it. There is no agreement on optimal culture conditions between different laboratories and even within the same laboratory. For *C. pneumoniae* to be a viable organism in the sense that it may be tested with regularity *in vitro*, a reliable method of culturing is required.

Molecular techniques, particularly PCR, are used to detect *C. pneumoniae* instead of culturing. However, in the case of antimicrobial testing this is not an alternative to culturing. This partly results from the fact that all the possible mechanisms of antibiotic resistance are not yet known. Therefore, testing for antibiotic resistance by investigating the presence or lack of certain genes is not appropriate. The absence of genes associated with resistance does not necessarily mean that the organism is sensitive to the antimicrobial agent. In contrast, the presence of a resistance gene does not necessarily mean that it is resistant. Only phenotypic testing such as MIC tests will indicate if an organism is sensitive or resistant. Thus we return to culturing.

Three different culture methods were investigated for their reproducibility using four different strains. TW 183, ATCC 2023 and AR 39 were collected from the American Type Culture Collection, the fourth strain, D 1, was received from Ninewells Hospital, Dundee. The D 1 strain had been successfully cultured using an in-house culture method in Dundee. All strains were stored at -70°C. HL (source: the Washington Research Foundation) and HEp-2 cells were used.

The first method was that of Sriram *et al* [3], the second was a method used by Roblin *et al* [4] and the third method was an in-house method used by Ninewells Hospital, Dundee, as described. A monolayer of HL cells were grown up in shell vials for 24 or 48 hours prior to inoculation. The growth medium was removed from the shell vials and the cells were rinsed with 1-2 mL of filter sterilised phosphate buffer solution (PBS). Each shell vial was inoculated with 300 µL of inoculum of TW 183, ATCC 2023, AR 39 or D 1. The vials were then centrifuged at 2400 X g and 35°C for 1 hour. The medium was replaced with medium containing 1.3 µg/mL cycloheximide. The vials were incubated at 37°C for 7 days with additional centrifugation on days 3, 4 and 5. On day 7 the

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vials were sonicated in an ultrasonicating water bath. 300 µL of the sonicated medium were added to freshly prepared monolayers. All negative controls were processed before examination of those thought to contain *C. pneumoniae*. Negative controls consisted of a shell vial containing uninoculated cells. The cultures were fixed and stained using direct and indirect antibody tests. No inclusions were found in any of the vials tested. The cells were washed and the growth medium and PBS were filtered in order to eliminate bacterial contamination. Three different methods with four different strains, two different cell lines and two different strains did not result in identification of a single *C. pneumoniae* inclusion. The lack of inclusions suggests that either the *C. pneumoniae* were not viable or the methods used are not easy to reproduce. As the D 1 strain had previously been cultured this strain was viable and so should have resulted in the identification of inclusions.

Thus none of the methods attempted could be reproduced. This lack of reproducibility is a common problem with *C. pneumoniae*, and should be highlighted. Development of new methods will not alter the problem of *C. pneumoniae* culturing. Standardisation of the existing methods so that they are reproducible not only in the same laboratory but also in different laboratories is the only way that data, particularly information about the antibiotic profile of *C. pneumoniae*, may be generated.

Standard, reproducible methods, which are agreed upon by more than one laboratory are needed not only for culturing but also for the investigation of antibiotic resistance in *C. pneumoniae*. A working party of *Chlamydophila* scientists is needed that will create culture guidelines with defined parameters similar to those available for antimicrobial agent testing. Thus accurate and valid information on culturing would be available.

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The Influence of the *erm* and *mef* genes on Telithromycin Resistance in *Streptococcus pneumoniae*.

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Objectives: To investigate the effects of the presence of *erm* and *mef* genes on the ability to select for telithromycin resistance in *Streptococcus pneumoniae*.

Methods: The *S. pneumoniae* strains chosen for investigation were 02J1095 containing the *erm* gene, 02J1175 with the *mef* gene and a macrolide sensitive strain NCTC 13593. Mutants were selected from plates containing telithromycin at the MIC or twice the MIC and subcultured twice on agar plates containing telithromycin. This procedure was repeated for each generation until telithromycin resistance occurred or successive generations resulted in the same MIC.

The *erm* and *mef* genes from the parent and the mutant strains were amplified using PCR and sequenced to investigate changes in the gene sequences from parent to mutant.

Results:

Strain	**Telithromycin MIC (mg/L)**	**Mutation frequency**
02J1095 (*erm*)	0.06	Parent
J I	1	1×10^{-3}
J II	>32	1×10^{-6}
02J1175 (*mef*)	0.5	Parent
M I	2	2×10^{-4}
M II	4	3×10^{-5}
M III	8	3×10^{-6}
M IV	8	3×10^{-5}
NCTC 13593	0.016	Parent

N I	0.032	2×10^{-2}
N II	0.12	3×10^{-2}
N III	0.5	6×10^{-1}
N IV	0.5	2×10^{-2}

The strains 02J1095 and 02J1175 were both sensitive to telithromycin although they were resistant to macrolides due to the presence of the *erm* and *mef* genes respectively. Telithromycin did not select for resistance in the macrolide sensitive NCTC 13593 strain after four generations of mutation. However, resistance to telithromycin occurred in both 02J1095 and 02J1175 mutants. Second generation 02J1095 mutants had high-level telithromycin resistance whereas the highest telithromycin MIC was 8mg/L after four mutant generations of 02J1175.
There were no nucleotide differences between the *erm* genes from the parent (02J1095) and the corresponding mutant strains. The *mef* genes from each of the four mutant 02J1175 generations also showed no changes to the parent 02J1175 *mef* gene.

Conclusions: Therefore, these results suggest that telithromycin does not select for resistance in strains lacking the *erm* or *mef* genes but will select for resistance in strains containing either gene. Thus in order to select for telithromycin resistance the strain must first be macrolide resistant due to an *erm* or *mef* gene. Also, high level resistance to telithromycin is selected with fewer generations in *S. pneumoniae* containing the *erm* gene rather than the *mef* gene.

Comparative *in vitro* Activity of Faropenem against *Streptococcus pneumoniae, Moraxella catarrhalis* and *Haemophilus influenzae* Isolated in the United Kingdom.

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Abstract

Faropenem is a new carbapenem. The agar dilution method was used to determine the susceptibility of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* to faropenem, a macrolide, a beta-lactamase inhibitor and four fluoroquinolones. The bacteria were collected from various regions of the United Kingdom. The *Streptococcus pneumoniae* (total n = 100) were collected from Edinburgh and Leeds, the *Haemophilus influenzae* (total n = 100) from Edinburgh and Glasgow and the *Moraxella catarrhalis* (total n = 100) from Edinburgh, Leeds and Wales. The *in vitro* tests were carried out in air incubators. The final inoculum for *Streptococcus pneumoniae* was 10^5 cfu per spot whereas *Haemophilus influenzae* and *Moraxella catarrhalis* were both 10^4 cfu. The antimicrobials tested were faropenem, amoxiclav, clarithromycin, moxifloxacin, levofloxacin, ciprofloxacin and gemifloxacin.

Antimicrobial Agents	*Streptococcus pneumoniae* Range MIC_{50}/MIC_{90} (mg/L)	*Moraxella catarrhalis* Range MIC_{50}/MIC_{90} (mg/L)	*Haemophilus influenzae* Range MIC_{50}/MIC_{90} (mg/L)
Faropenem	0.002-1 0.008/0.25	0.032-1 0.12/0.5	0.06-4 0.5/1
Amoxiclav	0.004-2 0.016/0.5	0.002-0.25 0.016/0.25	0.016-2 0.25/0.5
Clarithromycin	0.008->32 0.06/2	0.032-0.25 0.12/0.12	<0.06-16 4/8
Moxifloxacin	0.032-0.5 0.12/0.25	0.016-0.12 0.06/0.06	0.004-0.25 0.008/0.016
Levofloxacin	0.12-2 1/2	0.016-0.12 0.032/0.06	0.004-1 0.008/0.016
Ciprofloxacin	0.032-8 1/4	0.016-0.6 0.032/0.06	<0.004-2 0.008/0.016
Gemifloxacin	0.008-0.12 0.032/0.06	0.002-0.016 0.008/0.016	0.002-0.12 0.002/0.004

Streptococcus pneumoniae is the most susceptible of the three species to Faropenem. It has an MIC_{90} of 0.25 mg/L. For *Moraxella catarrhalis* and *Haemophilus influenzae* faropenem has MIC_{90} of 0.5 and 1 mg/l respectively. They are both one fold higher than amoxiclav.

For *Haemophilus influenzae* clarithromycin is poorly active whereas the quinolones exhibited strong activity. Levofloxacin and ciprofloxacin were moderately active against *S. pneumoniae*, but moxifloxacin and gemifloxacin had excellent activity. However, in contrast to beta-lactams quinolones cannot be used for treatment of paediatric infections or in pregnant women.
Faropenem has low MIC_{90} values for all three bacteria and might be a suitable alternative for treatment of respiratory infections caused by *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis*.

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THE *IN VITRO* ACTIVITY OF FAROPENEM AGAINST *STREPTOCOCCUS PNEUMONIAE*, *MORAXELLA CATARRHALIS* AND *HAEMOPHILUS INFLUENZAE* AND THE POTENTIAL FOR RESISTANCE DEVELOPMENT

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REVISED ABSTRACT

Faropenem is a novel member of the penem class of beta-lactams designed for the oral treatment of community-acquired infections.

Faropenem has good activity against Gram-positive bacteria. The agar dilution method was used to determine the susceptibility of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, isolated in the United Kingdom, to faropenem, a macrolide and a β-lactamase inhibitor. The *in vitro* tests were carried out in air incubators. The final inoculum for *Streptococcus pneumoniae* was 10^5 cfu per spot whereas *Haemophilus influenzae* and *Moraxella catarrhalis* were both 10^4 cfu.

Antimicrobial Agents	*Streptococcus pneumoniae*		*Moraxella catarrhalis*		*Haemophilus influenzae*	
mg/L	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Faropenem	0.008	0.25	0.25	0.5	0.5	1
Amoxiclav	0.016	0.5	0.016	0.25	0.5	1
Clarithromycin	0.06	8	0.12	0.12	4	16

To enable the rational development of new antimicrobials it is crucial that propensity for resistance selection is investigated as part of the development process.

As there were no significantly resistant bacteria in the clinical population, we attempted to generate resistant mutations in the laboratory using standard strains. The MIC of faropenem was determined in the absence and presence of CO_2. The strains were then challenged with increasing doubling concentrations above the MIC.

	With CO_2		Without CO_2	
	MIC	Mutation rate	MIC	Mutation rate
S pneumoniae NCTC13593	0.016	$<10^{-7}$	0.008	2.4×10^{-5}
*H. influenzae** NCTC1614	8	$<10^{-7}$	8	$<10^{-7}$
S aureus NCTC 6571	0.06	$<10^{-7}$	0.032	$<10^{-7}$
*S aureus**	0.06	1.1×10^{-6}	0.06	2.0×10^{-5}

*β-lactamase positive

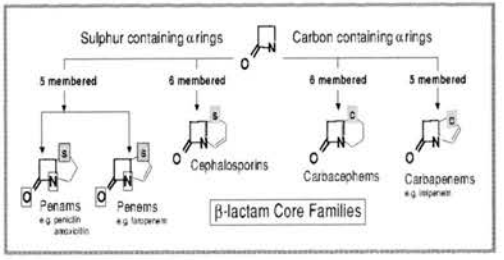
These results show that faropenem does not readily select resistance, even in β-lactamase positive strains, suggesting a decreased potential for resistance development in the clinical population.

INTRODUCTION

Respiratory infections are the most common cause of antibacterial prescribing (1). The selection pressure exerted by the use of these agents has resulted in the emergence of multiresistant isolates (2). In fact, recent surveillance data has clearly indicated an increase of resistant populations within the most common respiratory pathogens such as *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* (3). The need for therapeutic alternatives has provided the impetus for the recent development of extended spectrum quinolones and highlights the need for new β-lactams. Faropenem is a novel oral antibiotic with a penem structure unique from carbapenems and other available β-lactams, and designed for the treatment of community-acquired infections.

INTRODUCTION (continued)

Figure 1: Beta-lactam core family figure



Faropenem is characterized by high PBP affinity coupled with β-lactamase stability and demonstrates a spectrum which targets the gram-negative and positive bacteria commonly isolated from community infections (4). Therefore the initial aim of this study was to firstly evaluate the *in vitro* activity of faropenem against recently isolated clinical isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Secondly, the potential for resistance development with faropenem as the selecting agent was investigated in laboratory mutation studies.

MATERIALS AND METHODS

BACTERIAL ISOLATES

Streptococcus pneumoniae (100) from Edinburgh and Leeds, *M. catarrhalis* (100) from Edinburgh, Leeds and Wales and *H. influenzae* (100) from Edinburgh and Glasgow were used in this study. The control strains consisted of *S. pneumoniae* NCTC 13593, *Staphylococcus aureus* NCTC 6571, *H. influenzae* NCTC 11931 and a laboratory reference strain of *M. catarrhalis*.

MIC TESTING PROTOCOL

Minimum inhibitory concentrations were determined by standard agar dilution methods according to the BSAC method for sensitivity testing (5) on Columbia agar supplemented with 5% defibrinated horse blood for *S. pneumoniae* and *M. catarrhalis* and chocolate Columbia agar plates for *H. influenzae*. A final concentration of 10^5 cfu per spot of *S. pneumoniae* and 10^4 cfu per spot of *H. influenzae* and *M. catarrhalis* were inoculated onto each plate. Plates were incubated aerobically.

MUTANT SELECTION PROTOCOL

Stepwise mutants of each strain were selected by inoculating 10^8 cfu onto Columbia agar with the appropriate supplements containing faropenem at concentrations ranging from 1 to 4 times the MIC. Following incubation at 37°C in an atmosphere of 4 to 6% CO_2 for 3 days, mutants were then subcultured onto antibiotic free medium.

RESULTS

Table 1: *In vitro* activity of faropenem and comparators against respiratory isolates from UK centers

Antimicrobial Agents	*Streptococcus pneumoniae*		*Moraxella catarrhalis*		*Haemophilus influenzae*	
mg/L	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Faropenem	0.008	0.25	0.25	0.5	0.5	1
Amoxiclav	0.016	0.5	0.016	0.25	0.5	1
Clarithromycin	0.06	8	0.12	0.12	4	16
Levofloxacin	1	2	0.032	0.06	0.008	0.016
Ciprofloxacin	1	4	0.032	0.06	0.008	0.016

The MIC_{90}s of faropenem against *S. pneumoniae* were 2-16 fold lower than those recorded for amoxiclav and clarithromycin. Against *H. influenzae* the activity of faropenem was similar to amoxiclav. Clarithromycin was found to be the least active antibacterial against *H. influenzae*. In contrast, after the quinolones, clarithromycin was the most effective agent against *M. catarrhalis* followed by amoxiclav and faropenem. This rank order of activity is indicative of the β-lactamases harboured by the majority of clinical *M. catarrhalis* isolates.

Table 2: *In vitro* mutation rates obtained for faropenem

	With CO_2		Without CO_2	
	MIC	Mutation rate	MIC	Mutation rate
S pneumoniae NCTC13593	0.016	$<10^{-7}$	0.008	2.4×10^{-5}
*H. influenzae** NCTC1614	8	$<10^{-7}$	8	$<10^{-7}$
S aureus NCTC 6571	0.06	$<10^{-7}$	0.032	$<10^{-7}$
*S aureus**	0.06	1.1×10^{-6}	0.06	2.0×10^{-5}

*β-lactamase positive

The mutation studies were performed in both air and reduced air conditions (5% CO_2) with *S. pneumoniae* NCTC 13593 and *S aureus* NCTC 6571. Mutational studies were performed with faropenem at a range of concentrations between 1 and 4 times MIC. The mutational frequencies recorded for both strains in reduced air incubations are $<10^{-7}$. The presence of a β-lactamase in *S. aureus* does not appear to increase the mutational frequencies significantly in comparison to the strain without a β-lactamase (See Table 2). In normal air incubation, no differences in the mutational rates are recorded for both *H. influenzae* NCTC 1614 and *S. aureus* NCTC 6571 in comparison to the results in a reduced air atmosphere. The only increase in the mutation rates (two-fold increase) were observed with *S. pneumoniae* NCTC 13593 and *S. aureus* when incubated in air.

Generally, the mutation frequencies for all standard strains varied between $<10^{-7}$ and $<10^{-5}$. The low mutation values obtained here generally indicate the rarity of mutant development even in the β-lactamase positive strains.

CONCLUSIONS

- Faropenem has an MIC_{90} of ≤1μg/mL against *S. pneumoniae* *M. catarrhalis* and *H. influenzae*, which indicates that it ha significant activity against all three bacteria.
- It has the highest activity against *S. pneumoniae*, which is th main cause of community-acquired pneumonia.
- While the quinolones have lower MIC values than faropener for *Moraxella catarrhalis* and *Haemophilus influenzae* they ar not recommended for treatment of paediatric infections or i pregnant women.
- Low mutational frequencies observed indicate a minima potential for resistance development in *S. pneumoniae* *S. aureus* and *H. influenzae*.
- Faropenem warrants further investigation for the treatment o respiratory tract infections caused by *S. pneumoniae* *M. catarrhalis* and *H. influenzae*.

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Comparative *In Vitro* Activity of Telithromycin against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Hemophilus influenzae* Isolated in the United Kingdom.

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Abstract

Telithromycin (Tel) is a semisynthetic 14-membered ring macrolide derivative belonging to the ketolide family. The *in vitro* activity of Tel against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Hemophilus influenzae* was compared to that of a wide variety of antimicrobial agents.

The bacteria tested were collected from different areas of the United Kingdom: *Streptococcus pneumoniae* (n=100) from Edinburgh and Leeds, *Hemophilus influenzae* (n=100) from Edinburgh and Glasgow and *Moraxella catarrhalis* (n=100) from Edinburgh, Leeds and Wales. The susceptibilities of these organisms to the antimicrobial agents were tested using the agar dilution method. The *in vitro* tests for Tel utilised both ambient air and 5% carbon dioxide incubation, all other antimicrobial agents were tested in the ambient air.

Antimicrobial agents	*Streptococcus pneumoniae* MIC_{90} (mg/L)	*Moraxella catarrhalis* MIC_{90} (mg/L)	*Hemophilus influenzae* MIC_{90} (mg/L)
Telithromycin air	0.12	0.12	2
Erythromycin	8	0.25	16
Clarithromycin	4	0.12	16
Azithromycin	16	0.12	2
Clindamycin	0.12	4	16
Moxifloxacin	0.25	0.06	0.032
Gemifloxacin	0.06	0.016	0.004
Linezolid	2	8	16
Amoxicillin	0.5	4	4
Amoxiclav	0.5	0.12	1

Tel has one of the lowest MIC values of the antimicrobial agents tested and is consistently lower than erythromycin for *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Hemophilus influenzae*. Therefore, Tel has the potential to be a potent drug in the fight against respiratory tract infections.

Keywords: telithromycin, *Streptococcus pneumoniae*